
THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

# The role of Eph proteins in haematopoiesis and leukaemia

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*A thesis submitted for the degree of Doctor of Philosophy at*
*The University of Queensland in 2014*
*The University of Queensland School of Medicine*

# ABSTRACT

Eph and their membrane bound ephrin ligands represent the largest family of receptor tyrosine kinases (RTKs). Ephs interact with cell-surface ephrin ligands to direct cell migration and orchestrate developmental patterning during embryogenesis by modulating cell shape and adhesion. Members of the Eph/ephrin family of RTKs are expressed in adult life and have ongoing roles both in normal tissue homeostasis and in responses to pathological situations. Eph/ephrin proteins are also expressed on many tumours including leukaemia, prostate cancer, colorectal cancer and brain cancer. High expression in a number of cancers has been linked to progression through facilitation of invasiveness and metastatic spread.

Some members of the Eph/ephrin family of proteins are expressed on normal and cancerous haematopoietic cells. Real time PCR analysis showed expression of all EphA proteins on the haematopoietic stem cell (HSC) population, except for EphA6 and EphA8. I have used EphA1, EphA2 and EphA7 knockout mice to investigate the effect of these individual Eph proteins in normal haematopoiesis, and in particular haematopoietic stem cells *in vivo*. These studies showed that mice lacking EphA1, EphA2 or EphA7 have normal haematopoiesis and no significant defects were observed in HSCs. This suggests that if Eph receptors have a role in haematopoietic differentiation the presence of other Eph receptors may compensate for the absence of individual Eph proteins.

EphA2 expression has been investigated in many different solid tumours and expression of this member of the Eph/ephrin family has been detected in mixed lineage leukaemia (MLL). The role of EphA2 in leukaemogenesis was investigated in a syngeneic, retroviral model of MLL-AF9 induced acute myeloid leukaemia (AML). When HSCs from wild type mice were transduced with the MLL-AF9 retrovirus and engrafted into recipient mice, the subsequent leukaemias showed significant expression of EphA2 on leukemic blast cells. However when EphA2 null HSCs were engrafted, the absence of EphA2 appeared to have no effect on the leukemic process, demonstrating that EphA2 is not essential for leukaemia progression. Analysis showing increased expression of EphA7 and other Eph proteins demonstrate a possible compensatory role for the lack EphA2 expression, if Eph signalling was required for leukemic evolution in this model. Following these observations, the role

of EphA2 as a therapeutic target for MLL-AF9 leukaemia was evaluated. Results showed that radiolabelled (beta particle-emitting lutetium) EphA2 was an effective therapeutic approach by significantly delaying the progression of MLL-AF9 leukaemia. Given the poor prognosis of MLL-driven paediatric acute lymphoblastic leukaemia (ALL) this data raises the prospect of effective EphA2-targeted therapy for this disease.

EphA3 is another member of the Eph/ephrin family of RTKs, which was originally described in leukaemia and has subsequently been described in many other different cancers including sarcomas, lung cancer, melanoma and glioblastoma. The EphA3-specific monoclonal antibody, IIIA4, binds and activates both human and mouse EphA3 with similar affinities. High expression of EphA3 has been reported previously on LK63 pre-B ALL cell line which contrasts with lack of expression of EphA3 in the Reh, a similar pre-B ALL cell line. The effect of IIIA4 monoclonal antibody treatment on LK63 and Reh xenografts in SCID-NOD mice was examined, to explore both direct anti-tumour and potential effects of this antibody on the host cells. In this xenograft model of leukaemia, using the LK63 cell line, administration of the IIIA4 antibody led to inhibition of both tumour growth and spread from bone marrow to the spleen and other organs, hence increasing the latency of the disease. In contrast, there was no significant reduction in engraftment in the Reh xenograft model, suggesting that the anti-tumour effect was directed against the leukemic cells rather than the stromal and vascular elements.

To further analyse the therapeutic targeting of EphA3, LK63 EphA3-knockdown and Reh EphA3-expressing cells were tested in the xenograft model. Similarly to the LK63 model of leukaemia, the EphA3-expressing Reh xenograft model showed a reduction in bone marrow engraftment and slowing of disease progression upon treatment with EphA3 monoclonal antibody. Consistent with the effect of EphA3 monoclonal antibody treatment on Reh xenograft model, the LK63 EphA3 knockdown xenograft, showed minimal differences between treated and control group but had a notably significant reduction in the splenic engraftment compared to the normal LK63 model. A more impressive anti-tumour effect was delivered by antibody-mediated targeting of a radio-active isotope to EphA3-positive leukemic cells, in this case radioactive bismuth (alpha particle emitting) isotope-linked to IIIA4.

More importantly, no toxicity to normal tissues was observed, consistent with EphA3 being expressed at very low or being entirely absent on normal tissues.

In summary, in this thesis I have shown that whilst individual Eph receptors do not have critical roles in normal and malignant haematopoietic cells, individual Eph proteins may be candidates for targeted, antibody-based therapies in blood cancers.

# DECLARATION BY AUTHOR

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly authored works that I have included in my thesis.

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# PUBLICATION DURING CANDIDATURE

## Peer-reviewed papers

- Day BW, Stringer BW, Al-Ejeh F, Ting MJ, Wilson J, Ensbey KS, Jamieson PR, Bruce ZC, Lim YC, Offenhauser C, **Charmsaz S**, Cooper LT, Ellacott JK, Harding A, Leveque L, Inglis P, Allan S, Walker DG, Lackmann M, Osborne G, Khanna KK, Reynolds BA, Lickliter JD, Boyd AW. **EphA3 maintains tumorigenicity and is a therapeutic target in glioblastoma multiforme.** Cancer Cell. 2013 Feb 11;23(2):238-48.

- Day BW, Stringer BW, Spanevello MD, **Charmsaz S**, Jamieson PR, Ensbey KS, Carter JC, Cox JM, Ellis VJ, Brown LB, Walker DG, Inglis P, Allan S, Reynolds BA, Lickliter JD, Boyd AW. **ELK4 neutralization sensitizes glioblastoma to apoptosis through downregulation of the anti-apoptotic protein Mcl-1.** Neuro Oncology. (2011) 13 (11): 1202-1212

## Review

- **Charmsaz S**, Boyd AW. **Expression and Function of the Eph Receptor Family in Leukemia and Hematopoietic Malignancies: Prospects for Targeted Therapies**. Journal of Leukemia. (2013) 1:107

## Presentations

- **Charmsaz S**, Yeadon TM, Miller KJ, Lane SW, Ting MJ, Day BW, Boyd AW. **Regulation of hematopoietic stem/progenitor cells by Eph/ephrin signalling.** Keystone haematopoiesis meeting. 2011

- **Charmsaz S**, Yeadon TM, Lane SW, Miller KJ, Ting MJ, Stringer BW, Cooper L, Boyd AW. **The Role of Epha3 as a therapy target in leukaemia.** 41st Annual Scientific Meeting of the ISEH Society for Hematology and Stem Cells, Amsterdam, 2012.

- **Charmsaz S**, Day BW, El-Ajeh F, Palath V, Yarranton G, Bebbington C, Scott A, Lackmann M, Boyd AW. **EphA3 is a therapy target for leukaemia and other cancers.** 42nd Annual Scientific Meeting of the ISEH Society for Hematology and Stem Cells, Vienna, 2013.

- **Charmsaz S,** Lane SW, Boyd AW. **The role of EphA2 in haematopoiesis and leukaemia.** 42nd Annual Scientific Meeting of the ISEH Society for Hematology and Stem Cells, Vienna, 2013.

# PUBLICATIONS INCLUDED IN THIS THESIS

## Publication incorporated as part of chapter 1.

- **Charmsaz S,** Boyd AW. **Expression and Function of the Eph Receptor Family in Leukemia and Hematopoietic Malignancies: Prospects for Targeted Therapies.** Journal of Leukemia. (2013) 1:107

| Contributor | Statement of contribution |
|---|---|
| Sara Charmsaz (Candidate) | Wrote the paper (80%) |
| Andrew Boyd | Wrote and edited paper (20%) |

## CONTRIBUTION BY OTHERS TO THE THESIS

My supervisors, Prof. Andrew Boyd and Dr. Steven Lane made significant contributions to the project design throughout.

Chapter 2 - My overall contribution was 100% to the experiments described in this chapter. Professor Lynn Corcoran (Walter and Eliza Hall Institute of Medical Research) provided reagents and helped design the experiment on B cell development (Figure 2.5).

Chapter 3 - My overall contribution to this chapter was 95%. Dr. Fares.Al-Ejeh helped in the design and performance of the radiolabelled EphA2 antibody experiment included in chapter 3 (Figure 3.6).

Chapter 4 - My overall contribution to this chapter was 70%. Dr. Con Stylianou and Dr Trina Yeadon conducted preliminary studies of the LK63 model, which are included in part in (Table 4.1, Figure 4.2B and C, Figure 4.3 A, C and D) Dr. Andrew Scott and Dr FT Lee (Ludwig Cancer Research) helped design and conducted the experiment using the Bismuth labelled IIIA4 antibody (Figure 4.8 and Figure 4.9).

## STATEMENT OF PARTS OF THE THESIS SUBMITTED TO QUALIFY FOR AWARD OF ANOTHER DEGREE

None

# ACKNOWLEDGEMENTS

First and foremost, I owe my uttermost gratitude to my principle supervisor **Prof. Andrew Boyd**, for his incredible scientific insight, continuous support, guidance and patience. Thank you for your precious help, it has been a great experience learning from you.

To my associate supervisor **Dr. Steven Lane**, thank you for your support and guidance and thanks for teaching me specially in my initial years as a PhD student.
I am very grateful to **Dr. Claudia Bruedigam** for her help with the haematopoietic work. I would like to acknowledge all the past and present members of the leukaemia foundation laboratory especially **Fiona Smith, Leanne Cooper** and **Kirrilee Becket**.
I would like to acknowledge the leukaemia foundation for offering me a scholarship and giving me the opportunity to perform my PhD.

The biggest thanks go to my amazing family, it is impossible for me to put into words my gratitude and love towards them. To my **parents, Zahra and Mahmood Charmsaz** I am eternally grateful for all the sacrifices you have made for me. Thanks for sending me to Australia and for always supporting me and believing in me, I wouldn't have been able to achieve anything in my life without you guys. I would like to thank my brother, **Kamran Charmsaz** who has always been a role model for me and has encouraged me to achieve my goals. My special thanks goes to my other wonderful brothers **Mohammadreza and Alireza Charmsaz**, for their continuous supports and specially for looking after me in the past four years making sure I am eating food and staying healthy.

Finally, I would like to extend my gratitude to my husband **Amin Ahmadi,** I don't know how I could have done this without you. Thank you for always listening to me and for all your encouragements and advises. You have always been there for me even at midnights when you drive all the way to the lab to pick me up from work. Thanks for your love and support; this journey would have been much harder without you.

# DEDICATION

This thesis is dedicated to my beloved parents ***Zahra and Mahmood Charmsaz*** for their unconditional love, endless support and encouragement.

I also dedicate this thesis to my beautiful friend ***Fati Nekoui*** who lost her battle with cancer in 2010. Her lost was one of the reasons I choose to undertake my PhD in the field of cancer research.

*In Loving memory of Fati Nekoui*

## KEYWORDS

Haematopoiesis, Leukaemia, Haematopoietic stem cells, Eph, Therapeutic, Monoclonal antibody

## AUSTRALIAN AND NEW ZEALAND STANDARD RESEARCH CLASSIFICATIONS (ANZSRC)

ANZSRC code: 111201, Cancer Cell Biology, 70%

ANZSRC code: 111204, Cancer Therapy, 30%

## FIELDS OF RESEARCH (FoR) CLASSIFICATION

FoR code: 1112 Oncology and Carcinogenesis, 100%

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# LIST OF ABBREVIATIONS USED IN THE THESIS

AGM: Aorta-gonad-mesonephros

LT-HSC: Long-term HSCs

LMO2: LIM domain only 2

HSC: Haematopoietic stem cell

ST-HSCs: Short-term HSCs

MPP: Multipotent progenitor cells

CMPs: Common myeloid progenitors

CLPs: Common lymphoid progenitors

GMPs: Granulocyte-macrophage progenitors

MEPs: Megakaryocyte/erythrocyte progenitors

NK: Natural killer

BM: Bone marrow

KD: Knockdown

KO: Knockout

SCL/Tal1: Stem cell leukaemia/T-cell acute lymphoblastic leukaemia 1

LMO2: LIM domain only 2

GATA-2: GATA binding protein 2

MLL: Mixed-lineage leukaemia

Runx1/Aml1: Runt-related transcription factor 1/ Acute Myeloid Leukaemia 1

GM: Granulocyte macrophage

CSF: Colony stimulating factor

JAK: Janus kinases

STAT: Signal transducer and activator of transcription

IL: Interleukins

GM-CSF: Granulocyte-macrophage colony stimulating factor

G-CSF: Granulocyte- colony stimulating factor

EPO: Erythropoietin

TPO: Thrombopoietin

SCF: Steel cell factor

FLT3: Fms-like kinase 3

M-CSF: Macrophage colony-stimulating factor

SCID: Severe combined immunodeficiency disease

ALL: Acute lymphoblastic leukaemia

AML: Acute myeloid leukaemia

CLL: Chronic lymphocytic leukaemia

CML: Chronic myeloid leukaemia.

Eph: Erythropoietin-Producing Hepatocellular

EGF: Epidermal growth factor

FAK: Focal adhesion kinase

P13K: Phosphatidylinositol 3-kinase

Ras/MAPK: Ras/Mitogen-activated protein kinase

KLS: $Lin^{-}ckit^{+}sca1^{+}$

RT-PCR: Real-time polymerase chain reaction

MSC: Mesenchymal stem cell

CAN: Copy number alteration

LSC: Leukaemia stem cell

CRGs: Cooperative response genes

T-LBL: T-cell lymphoblastic leukaemia/lymphoma

HCT: Hematocrit

WBC: White blood cell

RBC: Red blood cell

FBS/PBS: Foetal bovine serum/phosphate-buffered saline

HOX: Homeobox proteins

MEIS1: Myeloid ecotropic viral integration site 1

GSK-3: Glycogen synthase kinase 3

HSP-90: Heat shock protein 90

GFP: Green fluorescent protein

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

# Chapter 1 . LITERATURE REVIEW

## 1.1 Haematopoiesis and Leukaemia

### 1.1.1 Introduction to haematopoiesis

Haematopoiesis is characteristically described as a hierarchical developmental process in which all lineages of blood are continuously produced throughout life. The process of haematopoiesis initially occurs during embryogenesis in the extra-embryonic yolk sac. The first site of haematopoiesis within the embryo is the aorta-gonad-mesonephros (AGM) region, pluripotent haematopoietic stem cells generated in this region are believed to seed to other organs including liver, spleen, lymph nodes and bone marrow. Haematopoiesis eventually ceases in other organs as the bone marrow develops into specialised niche that support haematopoietic stem cell (HSC) and becomes the primary site of haematopoiesis throughout adult life [1].

Haematopoietic stem cells (HSCs) give rise to all mature blood cells and comprises a small population of cells, constituting of up to 1 in 10,000 bone marrow cells and 1 in 100,000 blood cells in adults [2]. Haematopoiesis is a multi-step process in which HSCs give rise to the cells that undergo lineage commitment, maturation, proliferation and eventual migration from the bone marrow. HSCs are capable of exponential proliferation and in response to appropriate stimuli give rise to progenitors of all blood lineages. They require growth factors for their survival, proliferation, maturation and differentiation into mature blood elements [3]. In accordance with their role in maintaining haematopoiesis throughout life, these cells have the capacity to self-renew and the ability to give rise to all haematopoietic cell lineages termed multipotency. Self-renewal of the HSCs occurs in either symmetrical or asymmetrical cell divisions. In the symmetrical self-renewal the cells divide into two identical daughter cells both with HSC properties and thus expanding the population of HSCs, however in the asymmetric self-renewal one daughter cell keep the HSC property while the other will initiate differentiation and expansion and eventually produce mature blood cells [4-6]. All mature blood lineages and their progenitors originate from HSCs, self-renewing HSCs can be divided into long-term HSCs (LT-HSCs) with the most extensive self-renewing capacity, these cells give rise to short-term HSCs (ST-HSCs) with a lower self-renewal capacity. LT-HSCs and ST-HSCs can both generate multipotent progenitor cells (MPPs) with no self-renewal capacity and a multilineage differentiation capability [7-9]. These cells give rise to two

types of oligopotent progenitors known as the common myeloid progenitors (CMPs) and the common lymphoid progenitors (CLPs). CMPs generate the more restricted granulocyte-macrophage progenitors (GMPs), which develops in to mature granulocytic or monocyte macrophage cells, and megakaryocyte/erythrocyte progenitors (MEPs), which can terminally differentiate into either megakaryocytes or erythrocytes [10,11]. CLPs will differentiate into progenitors of the mature B-lymphocytes, T-lymphocytes and natural killer (NK) cells [12]. Both CMPs and CLPs have been proposed to give rise to distinct subsets of dendritic cells (Figure 1.1) [13].

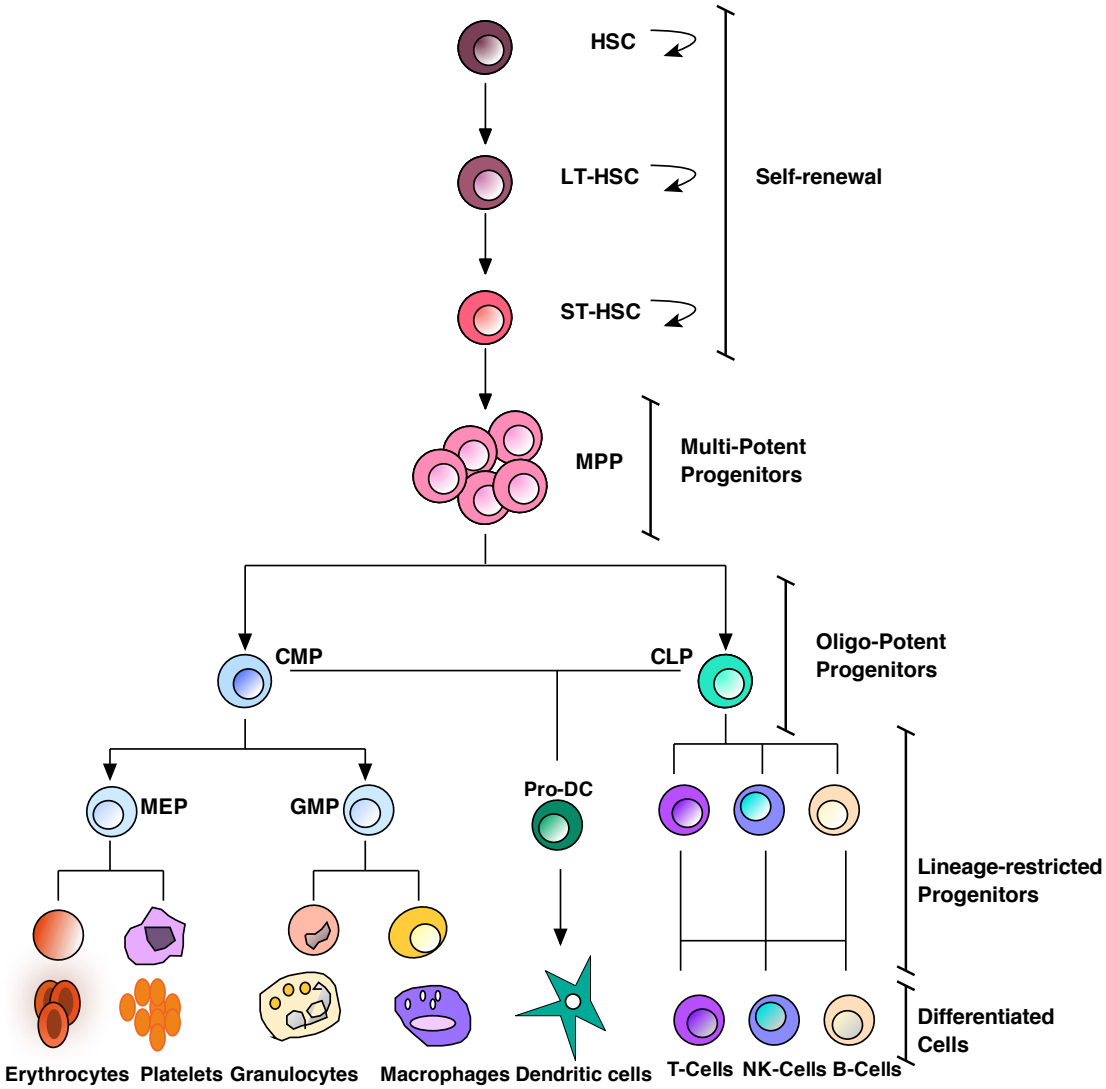



**Figure 1.1. Haematopoietic heirachy (Adapted from [13]).**

### 1.1.2 Transcription factors in haematopoiesis

Several factors are responsible for maintaining haematopoietic stem cell homeostasis, including the balance between self-renewal and differentiation, loss through apoptosis and migration to other sites. Regulation of self-renewal, differentiation and migration within the haematopoietic system is controlled by a variety of both extrinsic and intrinsic factors. Transcription factors are important intrinsic regulatory factors in the haematopoietic system, governing stem cell self-renewal and lineage commitment decisions through regulation of gene expression [1].

Some transcription factors, including stem cell leukaemia/T-cell acute lymphoblastic leukaemia 1 (SCL/Tal1), LIM domain only 2 (LMO2) and GATA binding protein 2 (GATA-2), are essential for both primitive (embryo-restricted) and definitive (adult) haematopoiesis, while others including mixed-lineage leukaemia (MLL) and runt-related transcription factor 1/ Acute Myeloid Leukaemia 1 (Runx1/Aml1) are necessary only for definitive haematopoiesis and therefore they are essential for the survival and proliferation of HSCs [14].

The SCL/Tal1 transcription factor is critical for initiation of haematopoiesis during early stages of foetal development [15]. It is also important but not essential for the development and function of adult HSCs and all blood cells, SCL1/Tal is also involved in specification of the erythroid and megakaryocytic lineages [16]. The LMO2 transcription factor is essential for both primitive and definitive haematopoiesis and mice lacking either SCL/Tal1 or LMO2 die in embryonic development due to a lack of haematopoietic progenitor cells in the early embryo, thus no blood is generated leading to embryonic loss [1]. LMO2 is also important in regulation of lineage specification in erythropoiesis [16].

GATA-1 and GATA-2 are both important definitive and primitive factors in haematopoiesis and loss of either of these can lead to embryonic lethality. In normal haematopoiesis GATA-1 is necessary for erythroid and megakaryocytic differentiation and GATA-2 is required for development of mast cells and regulation of HSCs [17,18]. Runx1 is required for development and function of HSC and Runx1 knockout mice lack definitive haematopoiesis and die during the mid-gestational period [19,20]. MLL is another transcription factor with an important role in early haematopoietic development [21], it has been reported that this gene lies upstream of

HOXB4 and is therefore involved in regulation of the HoxB4 gene, which is needed for specification of HSCs [1].

A number of other transcription factors, including Ikaros, activated nuclear form of Notch1, cell cycle inhibitor P21, members of the TGF/BMP-4 family, TNFα receptor and p55, are also involved in HSC survival and self-renewal [1,14]. Some of the transcription factors, including PU.1, C/EBP and GATA-1, have the ability to "reprogram", resulting in a switch of cell lineage when they are over expressed in the cells with a specific lineage fate [22]. In summary, the above transcription factors are important intrinsic regulators of HSCs and also in determining the pathway of lineage commitment [14] (Figure 1.2).

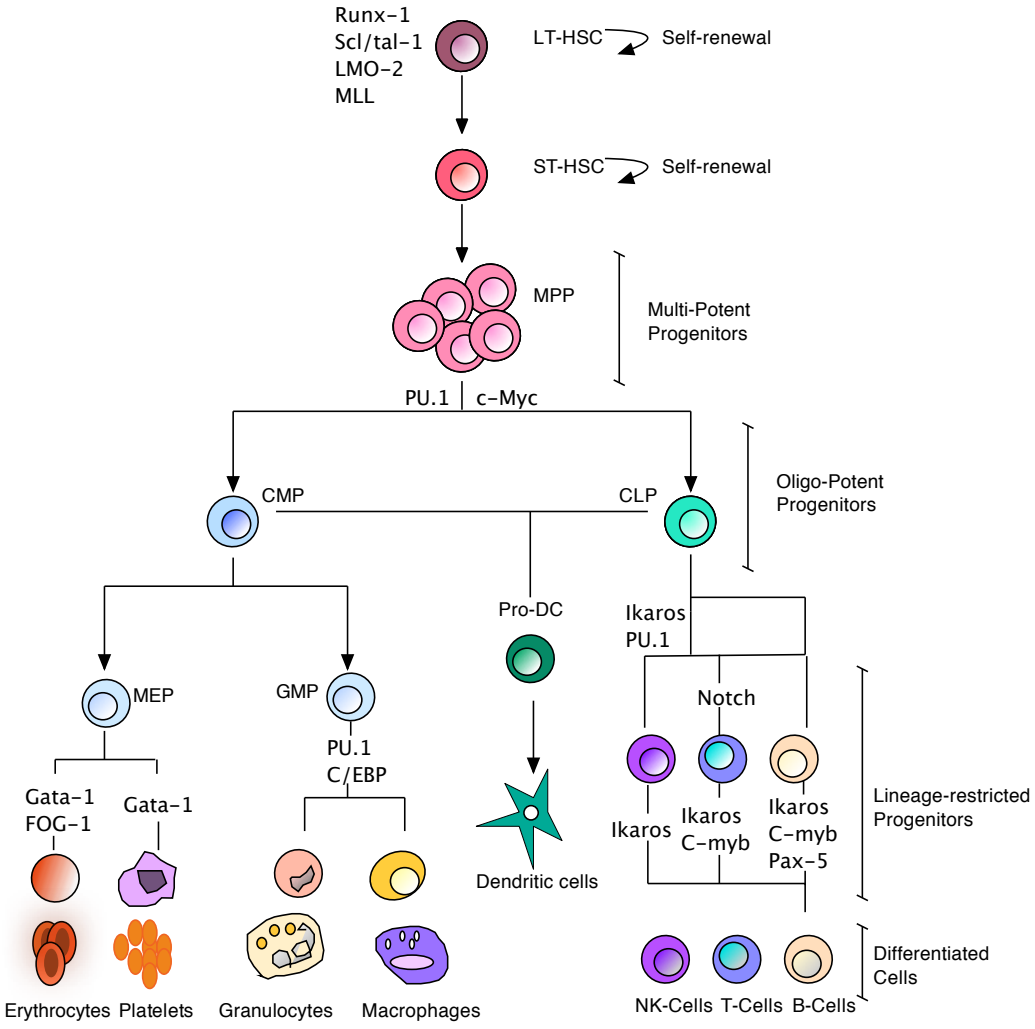



**Figure 1.2. Transcription factors involve in haematopoiesis (Adapted from [1,13]).**

### 1.1.3 The extrinsic regulators of HSC

Cytokines are a group of regulatory proteins with important functions during haematopoiesis and in blood cell function. They are involved in regulation of quiescence, self-renewal, differentiation, apoptosis, and cell migration, partly by inducing alterations in gene transcription [23]. Cytokines work by binding to specific hetero-dimeric cytokine receptors (e.g. Granulocyte-macrophage colony stimulating factor (GM-CSF) binds to the GM-CSF receptor a/b complex) and thus triggering the receptor to initiate a process, which mediate the downstream cellular response. In particular, the cytokine receptors signal by activation of Janus kinases (JAK), which are members of the tyrosine kinase family. These in turn activate downstream molecules and in particular signal transducer and activator of transcription (STAT) proteins [24].

Several cytokines act on progenitor cells and influence differentiation, proliferation, survival and functional activation of HSCs. Some of these cytokines are interleukins (IL-3, IL-5, IL-6, etc), granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor G-SCF [25]. Other important cytokines are erythropoietin (EPO) and thrombopoietin (TPO), which are members of the IL-2 subfamily. IL-3 is one of the most important members of interleukin family in early haematopoietic cell function; it is involved in survival, proliferation, differentiation and maturation of HSCs [26]. GM-CSF drives differentiation towards the granulocyte and macrophage lineages and G-CSF has a specific role in the production of neutrophils [27]. Thrombopoietin (TPO), a ligand for the c-mpl cytokine receptor, is the primary regulator of megakaryocyte differentiation and functions as a modulator of platelet production and release [28]. Erythropoietin (EPO) binds to the erythropoietin receptor and stimulates red blood cell development [29].

Receptor tyrosine kinases (RTKs) also mediate extrinsic signals that regulate haematopoiesis. Stem cell factor or steel cell factor (SCF) is a cell bound protein expressed by stromal cells, which binds to the c-kit receptor tyrosine kinase, expressed on HSC. This interaction has a key role in the survival, mobility and possibly self-renewal of HSCs. Fms-like kinase 3 (FLT3) is a member of the PDGFR family of RTKs and is involved in the development of multipotential stem cells and B cells. Macrophage colony-stimulating factor (M-CSF/CSF-1), binds to the c-fms

RTK, and is another lineage specific cytokine important in differentiation of monocyte progenitors and regulation of mature macrophages [30]. Members of the Eph family of RTKs, and their ephrin ligands, are also expressed in haematopoiesis and their role will be discussed later in this review.

### 1.1.4 The bone marrow niche and regulation of HSCs

Bone marrow is the main site of haematopoiesis in adults and within the bone marrow interactions with HSCs regulate the maintenance, proliferation, differentiation and migration of the cells into the blood stream. As alluded to in discussion of extrinsic factors, the fate of the HSC is controlled by influences within specific niches in the bone marrow microenvironment. There are currently believed to be two distinct niches, defined anatomically and physiologically. The endosteal region, or osteoblastic niche, is composed of non-haematopoietic bone-forming cells and other mesenchymal-derived stromal cells such as reticular cells, fibroblasts and adipocytes. This niche is remote from the vasculature and thus is relatively starved of oxygen, consequently it is often described as the hypoxic niche; this site has properties, which maintain the haematopoietic stem cells in a quiescent or dormant phase of the cell cycle. The other bone marrow HSC niche is known as the vascular niche. HSCs within this peri-vascular niche are close to the outer surface of the vascular sinusoids and thus can proliferate, differentiate and migrate through the sinusoidal wall.

In summary, whilst these functions form a continuum, the hypoxic, osteoblastic niche is believed to be biased towards maintaining the undifferentiated LT-HSCs while the vascular niche is more involved in differentiation and release of the mature HSCs [5,6,31,32]. Whilst most of the LT-HSCs in the bone marrow remain in the osteoblastic niche and in the G0 phase of the cell cycle, upon receiving the appropriate signal they will enter the cell cycle and divide, which result in differentiated, self-renewal and migration out of the niche into surrounding bone marrow and the general circulation [33].

### 1.1.5 Leukaemia

Leukaemia is a malignant disease of the haematopoietic system characterized by the abnormal proliferation of either uncommitted or partially committed HSCs [34]. Leukaemia is classified based on clinical aggressiveness and other features into two

broad groups: acute and chronic leukaemia. Both of these groups are then sub-classified, according to the origin of the leukemic cells, into myeloid or lymphoid leukaemia thus, leukaemia is classified into four main groups comprising acute lymphoblastic leukaemia (ALL), acute myeloid leukaemia (AML), chronic lymphocytic leukaemia (CLL) and chronic myeloid leukaemia (CML). The individual types (except CML) can be categorised more extensively based on similarities to individual types of lymphoid (eg pre-B ALL and T-ALL) and other haematopoietic cells (eg erythro leukaemia and promyelocytic leukaemia).

In many human leukaemias, the disease is caused by disruption in the regulation of normal haematopoiesis through altered or aberrant function of genes involved in regulation, self-renewal and apoptosis of HSCs. Different types of genetic alteration are responsible for development of leukaemia, including chromosomal translocation, epigenetic changes and mutations. Of these genetic lesions, alterations in genes responsible for proliferation and survival of the HSCs, and genes that regulate differentiation or self-renewal of the haematopoietic cells during development can give rise to leukaemia [35]. A prime example of these genetic alterations is BCR-ABL in which chromosomal translocations occur between chromosomes 9 and 22 resulting in formation of abnormal fusion proteins where the BCR-ABL fuses the Bcr and Abl kinase genes resulting in formation of a unique Bcr-Abl protein in which the Abl kinase is constitutively active, the end result is hyper-proliferation and altered maturation which manifest as CML [36]. Similarly, balanced translocation in the mixed-lineage leukaemia gene (MLL) result in expression of fusion proteins and the consequent altered gene regulatory disruptions leads to either ALL or AML leukaemia depending on the type of the translocation. For example the MLL-AF4 translocation is most frequently found in ALL, while the MLL-AF9 fusions is more common in AML [37].

## 1.2 Eph-ephrin

### 1.2.1 Classification, structure and binding of Eph/ephrin

The Eph receptors and their membrane bound ephrin ligands represent the largest family of receptor tyrosine kinases (RTK's). The first member was originally termed Eph, as it was first identified in an Erythropoietin-Producing Hepatocellular (Eph Nomenclature committee, 1997) carcinoma cell line [38]. In mammals there are fourteen

members of the Eph family of RTKs these are divided into two groups, the EphA and EphB family. This classification is based on their sequence homology, ligand specificity and structural features. In mammals there are nine members of the EphA subgroup (EphA1-8 and EphA10) and five EphB receptors (EphB1-4 and EphB6) [39,40].

Eph receptors are type I transmembrane proteins composed of an extracellular region with four distinct domains. An N terminal ligand-binding domain with two distinct ligand-binding interfaces (N terminal β jelly roll domain) determining the ephrin binding. This is followed by a cysteine-rich region, which contains an epidermal growth factor (EGF)-like motif involved in receptor dimerisation. The main domain is followed by two fibronectin type-III domains [41,42]. The intracellular region comprised of a conserved juxtamembrane domain, a tyrosine kinase domain and a sterile alpha motif (SAM) domain, the latter have a potential role in receptor clustering [43,44]. Most of the Eph receptors possess a C-terminal PDZ binding motif (Postsynaptic density protein, Disc large, Zona occludens tight junction protein) that is involved in signalling at a sub-cellular level and in the assembly of large molecular complexes [40,42,45] (Figure 1.3A).

The ephrin ligands of Eph receptors are membrane bound proteins and like the Ephs they are divided into two groups based on their structural features and preferential binding to either EphA or EphB receptors. The two classes of ephrin ligands are the A-class (ephrinA1-5) and B-class (ephrinB1-3) [40]. These two classes of the ephrin ligands have homology at their N-terminus region while their C-terminal amino acid sequence differs. The ephrinA ligands are bound to the plasma membrane by a glycosylphosphatidylinositol (GPI)-linker and the ephrinB ligands possess a transmembrane–spanning region and a highly conserved cytoplasmic tail with a number of highly conserved tyrosine residues and a PDZ-binding motif. The conserved tyrosine residues have been shown to serve as a docking site for proteins, which mediate downstream ephrin signalling (Figure 1.3B) [39,46].

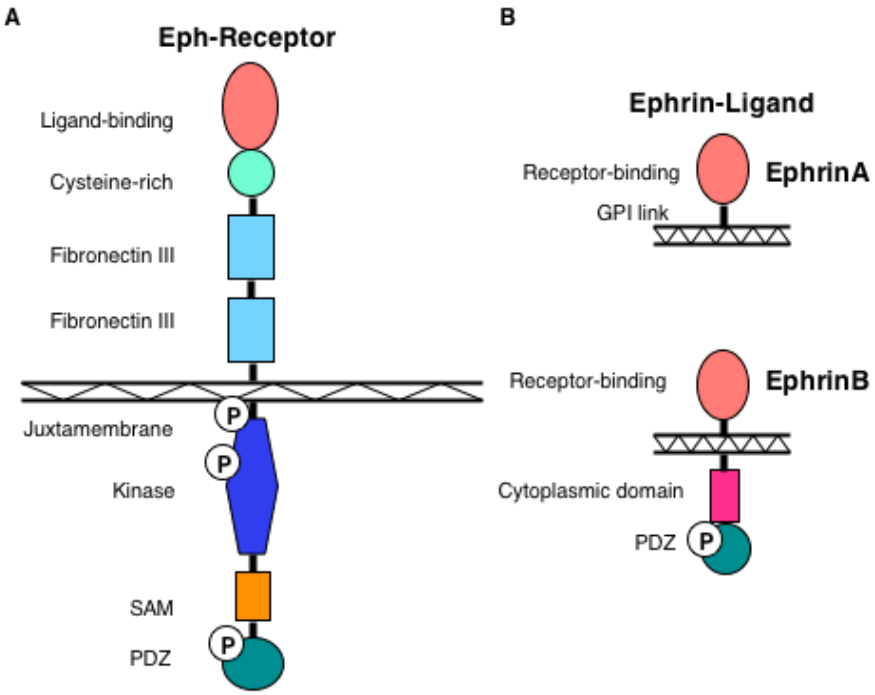



**Figure 1.3. Schematic representation of Eph receptors and ephrin ligand structure.** (A) The structure of the Eph receptor. (B) The structure of the ephrinA and ephrinB ligand.

Generally, the EphA receptors will preferentially bind to members of the ephrinA ligands (ephrinA1-5) while the EphB receptors will preferentially bind to the ephrinB ligands (ephrinB1-3) [40,47]. There is a high level of binding promiscuity between the Eph receptors and the ephrin ligands, however for an individual Eph receptor there is a distinct order of affinity of ephrin interactions with affinity constants ranging from 5-500 nM [48]. For instance, the EphA1 receptor binds with a high affinity to ephrinA1 and a lower affinity to other members of the ephrinA family, including ephrinA3 and ephrinA4, and shows essentially no binding to ephrinA5 [49]. Similarly EphA3 and EphB4 have a higher affinity for ephrinA5 and ephrinB2 respectively than for other members of the ephrin family [48,50]. High affinity interaction is also possible between the classes, for example EphA4 binds to both ephrinB and ephrinA ligands and some of its most important functions depend on interaction with ephrinB3. Another example is EphB2, which binds to ephrinA5 as well as ephrinB ligands (Figure 1.4) [51,52].

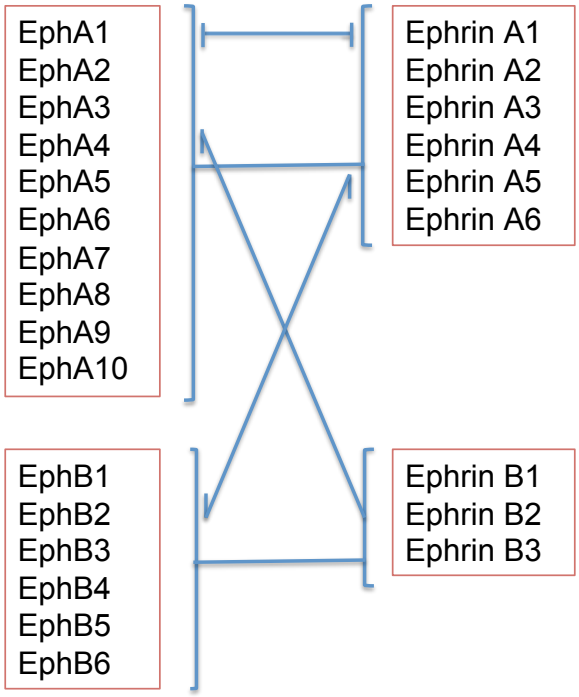



**Figure 1.4. Schematic of Eph receptor and ephrin binding preference.**

### 1.2.2 Eph/ephrin activation and signalling

Eph/ephrin interactions have the capacity to initiate bidirectional signalling. In other words both Ephs and ephrins can act as a ligand as well as a receptor and they both have the ability to initiate signalling. Signalling initiated by the Eph receptor is referred to as forward signalling whereas signalling initiated by the ephrin ligand is termed reverse signalling.

Eph receptor activation occurs following the physical association of the receptor and an ephrin ligand on adjacent cell surfaces mediated by the high affinity binding site (dimerization), this is followed by interaction with a second Eph/ephrin complex, mediated by separate low affinity (tetramerization) binding site to create a hetero-tetrameric complex [53]. The tetramers are then assembled into higher order clusters, which appear to be required for effective forward signalling [54,55]. The clustering of Eph receptors results in ligand-dependent auto-phosphorylation of several tyrosine residues within the cytoplasmic kinase domain and juxtamembrane region of the receptor, which serve as docking sites for downstream signalling proteins including the small GTPases of the Rho and Ras family, focal adhesion kinase (FAK), the janus

kinase/signal transducers and activators of transcription (Jak/Stat) and phosphatidylinositol 3-kinase (P13K) pathways [56].

Eph receptor activation influence cell shape and motility through the regulation of the Rho GTPases: including RhoA, Cdc42, Rac and Ras, via interactions with specific Rho GTPase activating (Rho GAP) and exchange (Rho GEF) factors. The interaction with individual Rho GTPases, mediated via direct binding to the cytoplasmic domain of Eph proteins of Rho GAP and Rho GEF or their recruitment via adaptor proteins, thereby mediating different effects on actin dynamics and cell process formation thus regulating cell shape and movement. For example, activation of EphA4 receptor results in ephexin (Rho GEF)-mediated activation of RhoA and α-chimaerin (Rho GAP)-mediated inactivation of Rac, resulting in actin depolymerization and retraction of cell processes [52]. Activation through EphB plays a role in actin filament extension, morphogenesis and maturation of dendritic spines. Ras family of GTPases (H- and R-Ras) are also activated through Eph receptors, however unlike the Rho GTPases, the majority of Eph receptors negatively regulate the Ras/Mitogen-activated protein kinase (Ras/MAPK) pathway, with activation normally resulting in regulation of proliferation and migration [52].

Eph receptors are also important in mediating a number of other molecules involved in cell migration and adhesion due to their ability to regulate signal transduction molecules including integrin signalling pathway elements paxillin, FAK, P130 (Cas) and integrins themselves. The effect of Eph signalling on integrins is complex as they can mediate either promoting or suppressing effects. FAK is important in mediating integrin signalling and Eph receptors can down-regulate this pathway [57].

The Jak/Stat pathway, involved in cell growth and viability, is regulated by EphA receptor activation [58]. EphB receptors mediate cell migration and proliferation through P13K pathway and the protein kinase B/phosphatidylinositol 3-kinase (Akt/ PI3K) pathway is involved in the regulation of cell proliferation and viability. Eph/ephrin also regulates other signalling pathways including Abl/Arg and p53-family of tumour suppressor proteins [56]. A recent study in glioblastoma multiforme (GBM) shows that loss of EphA3 results in elevated MAPK signalling thereby inducing differentiation and reducing proliferation and self-renewal. This study showed that regulation of extracellular signal-regulated kinases/mitogen-activated

protein kinases (ERK/MAPK) signalling by EphA3 is kinase independent of the upstream activators of MAPK signalling [59]. A similar finding has been made for GBM, which express EphA2 at high levels [60].

In the ephrin-expressing cells, reverse signalling can be induced after Eph/ephrin interaction [56,61]. EphrinB reverse signalling partly depends on the tyrosine phosphorylation of conserved residues in the cytoplasmic region, where phosphorylation is mediated by associated tyrosine kinases, most notably members of the Src family (Src, Fyn, Lyn, Yes) of tyrosine kinases. When the ephrinB ligand is phosphorylated, it can bind to cytosolic adaptor molecules via (Src Homology-2) SH-2 and SH-3 domains or PDZ domains [45]. The protein tyrosine phosphatase basophil-like (PTP-BL) has been identified as a negative regulator of ephrinB signalling and it binds to ephrinB through its C-terminal PDZ binding motif. De-phosphorylation of the ephrinB cytoplasmic domain can inactivate the Src family of kinase and therefore cause termination of reverse signalling [62]. In the activated ephrinB ligand, the PDZ motif plays an important role in assembly of other signalling molecules. EphrinB binds to cytoplasmic protein PDZ-RGS3, which contains a PDZ domain and a regulator of G-protein signalling (RGS) domain. Activation of the ephrinB ligand by EphB receptor via the PDZ-RGS interferes with signalling of the stromal derived factor (SDF)-1 via its G-protein coupled receptor and the chemokine receptor-4 (CXCR4). EphrinB reverse signalling has thus been implicated with regulation of migration in cerebellar development [63].

The ephrinA reverse signalling mechanism is not well understood, however it is likely that the signalling response is initiated by activity of the Src family of RTKs in which transmembrane adaptor molecules, associating with the lipid anchor of the ephrinA proteins, transmit the signal across the membrane. For example, ephrinA5 induces signalling within the ephrinA expressing cell when bound to its cognate Eph receptor [64-66].

### 1.2.3 Expression and function of the Eph and ephrin genes

Eph signalling controls cell adhesion, migration, invasion and morphology by influencing integrin and intercellular adhesion molecule activity and by modification of actin cytoskeleton organization as described above. Through these mechanisms Eph function effects not only the processes of embryogenesis but also specialised

cellular function in adult tissues, including bone remodelling, immune function and synaptic plasticity as well as cell proliferation and survival of specific tissue stem cells as described further below [64,67].

Both Eph and ephrin proteins are important in regulating cell-cell interactions and their interaction can initiate either cell adhesion or repulsion. Cell repulsion occurs when bidirectional signalling triggers cytoskeletal contraction, loss of focal adhesions, cell rounding and cell detachment, whereas cell attachment occurs when signals are in favour of cell adhesion and migration [68]. This interaction influences many different cell behaviours during embryogenesis and in adult life [64]. Eph/ephrin interactions mediate formation of tissue boundaries (e.g. hindbrain rhombomeres) [69], control axon guidance during development and also tissue morphogenesis and patterning [70,71]. Eph/ephrin interactions are also involved in development of vascular system [72], stem cell biology [73], haematopoiesis, erythropoiesis, immune function and in tumour invasion and metastasis [64,67,74-76].

Many members of the Eph/ephrin family are expressed at high levels in some cancer cells and also elements of the tumour microenvironment, where they influence tumour growth and spread. Specific examples include lung, breast and prostate cancer, as well as melanoma, sarcomas and leukaemias [77,78]. There is evidence that the Eph receptors can have either tumour suppressing or tumour promoting activity, depending on the tissue and their expression pattern [64]. Thus, the role and function of Eph/ephrins in cancer is not yet fully elucidated as some tumours show an elevated level of Eph expression while others show a decrease in Eph expression and as yet no single model of their function encompasses all cancers. For example, EphA2 is up-regulated in many cancers including breast and prostate and its expression is linked to an increase in malignancy [79,80], however it's is down-regulated in colon cancer [81]. Similarly, EphA1 expression is up-regulated in ovarian cancer [77] but down-regulated in advanced skin and colorectal cancers [82,83]. Studies have also shown the role of EphA7 as a tumour suppressor in follicular lymphoma [84], tumour suppressor function has also been reported for EphB receptors, including EphB2 and EphB3 in colorectal cancer [67,85]. EphB4 is another important example as this gene can act as either a tumour suppressor or an oncogene in different facets of breast cancer progression [86,87]. Table 1.1 represents the expression of Eph receptor in normal and malignant tissues.

**Table 1.1. Eph expression in normal and malignant tissues.**

| Eph | Tissue expression | Expression in cancer | References |
|---|---|---|---|
| EphA1 | Widely express in mouse epithelial tissues evidence of expression in hematopoietic progenitors | Over expressed in many different cancers including hepatocellular, colon, prostate, lung, gastric, colon cancer<br>Down regulated in non-melanoma skin cancer, colorectal cancer | 38,61,82,83,88-93 |
| EphA2 | Expressed highly in adult human epithelial cells and endothelium | Over expressed in prostate, breast, melanoma, lung and ovarian cancers<br>Up regulated in glioblastoma<br>Down-regulated in colon cancer | 60,61,79,80,92-99 |
| EphA3 | Expressed in various stages of embryonic development and in adult central nervous system | Expressed in neural cancers, leukaemia, lymphomas and sarcomas<br>Up-regulated in lung, brain, liver and kidney and over expressed in melanoma | 61,92,93,100-104 |
| EphA4 | Expressed in development, final stages of embryogenesis and central nervous system | Expressed in prostate, pancreatic cancer<br>Up regulated in lung cancer<br>Down-regulated kidney<br>Over expressed in gastric cancer | 61,92,93,100,105-108 |
| EphA5 | Expressed in nervous system | Expressed in neuroblastomas and neural cancer<br>Down-regulated in breast cancer | 61,92,93,108-110 |
| EphA6 | Expressed more prominent in adult tissues than in embryonic tissues | Down-regulated in colon cancer and renal carcinoma<br>Up-regulated in lung and liver cancer | 61,92,93 |
| EphA7 | Expressed in developing neural tubes, thymus, lymphoid tissues and foetal bone marrow | Expressed in colorectal cancer, lung, suppressed on follicular lymphoma<br>Up-regulated in ALL1 leukaemia | 61,81,84,92,93,111-113 |
| EphA8 | Expressed in spinal cord and neuronal cells | Expressed in colon cancer<br>Down regulated in glioblastoma | 61,92,93 |
| EphA10 | Expressed in testis | | 114 |
| EphB1 | Expressed in Brain and colon | Expressed in lung cancer<br>Down-regulated in colon carcinoma and in kidney cancer | 61,83,92 |
| EphB2 | Expressed in epithelial cells, thymus, lymphoid, osteoblastic and osteoclastic cells | Over expressed in gastrointestinal<br>Expressed in colon, ovarian and lung cancer<br>Up-regulated in colorectal, kidney and hepatocellular cancer | 61,92,115 |
| EphB3 | Expressed in various tissues | Expressed in prostate cancer, lung cancer and melanoma | 61,92 |
| EphB4 | Expressed in placenta and in range of primary tissues including brain, endothelium, hematopoietic cells | Expressed in colon cancer, endometrial, breast, neuroblastoma, glioblastoma and leukaemia and lymphoma cancer | 61,92,116-118 |
| EphB6 | Expressed in various tissues including brain, pancreas, thymus and T-cells | Expressed in thymus and leukaemia<br>Up regulated in colon cancer<br>Down-regulated in breast, lung and kidney cancer | 61,92,119 |

## 1.3 The Eph/ephrin in HSC and leukaemia

### 1.3.1 Eph/ephrin expression in HSCs and progenitors

The expression of Eph/ephrin has been detected on purified population of haematopoietic stem cells (HSCs) in both human and mouse. Gene expression analysis of HSCs showed expression of ephrinB2, indicating that it may be involved in signalling between HSCs and their microenvironment. Other array based studies on primary human HSCs ($CD34^+$ hematopoietic cells) showed expression of the EphA1 protein and its ligands ephrinA3 and ephrinA4 suggesting that their interaction may play a role in haematopoietic stem and progenitor cell positioning and function [120,121]. Further analysis of $CD133^+$ and $CD34^+$ hematopoietic stem cells in peripheral blood showed expression of EphA2 in all $CD34^+$ cells and the majority of $CD133^+$ cells however EphB2 was expressed in all $CD133^+$ cells and fifty percent of $CD34^+$ cells, these data suggest that a number of elements of the Eph/ephrin system may have a role in HSC function through regulatory effects on cell adhesion, migration and differentiation but also that there may be a degree of functional redundancy [122].

Real-time quantitative PCR of mouse $Lin^-ckit^+sca1^+$ (LKS) cells showed detectable expression of all EphA receptors except EphA6 and EphA8, along with ephrinA ligands, with the ephrinA4 and ephrinA5 being the most highly expressed ligands on purified HSCs in the mouse bone marrow [123]. Flow cytometric analysis of EphA2, A3, A4 and A5 along with ephrinA1-5 showed that EphA2 and EphA3 were the highest expressing EphA receptors. Expression of EphA2, A3, A4 and A5 was also detected in the mouse stromal cell lines however human stromal cell line expressed only EphA2 at moderate levels [123]. Whilst the function of Eph/ephrin has not been fully investigated, a role in HSC trafficking was demonstrated by treatment of mice with an Eph/ephrin inhibitor, EphA3-Fc, which resulted in mobilisation of bone marrow progenitor cells into peripheral blood [123].

Some of the members of the Eph/ephrin family are also involved in development and regulation of mature haematopoietic cells. For example, EphA4 and EphB1 along with ephrinB1 ligand are expressed on human platelets [124], these studies shows that EphA4 is involved in regulation of platelet aggregation and adhesion to fibrinogen, a process dependent on integrin $\alpha IIb\beta 3$ engagement [76].

EphB4 was originally identified on human bone marrow $CD34^+$ cells and its expression has been reported on erythroid progenitor cells in early stages of red blood cell development. Significantly, the EphB4 ligand, ephrinB2, is expressed on bone marrow stromal cells [125] where it has been reported to be involved with regulating erythropoiesis via interaction with EphB4 [126]. Studies by Suenobu et al [126] showed co-culturing haematopoietic progenitor cells expressing EphB4 with stromal cells expressing ephrinB2 results in haematopoietic progenitor cells detachment from stromal layer and differentiation into a mature erythroid cells accompanied by EphB4 down regulation, however co-culturing these cell with ephrinB2-negative stromal cells resulted in less maturation of erythroid cells and no change to EphB4 expression [126]. EphrinB2 ectopic expression in stromal cells increased adhesion of haematopoietic cells to stromal cells and decreased transmigration of haematopoietic cells beneath a stromal cell monolayer. These findings strongly support a role for the EphB4/ephrinB2 interaction in migration and colonisation of stem/progenitors cells in the bone marrow microenvironment [127].

Some of the Eph/ephrin molecules are also involved in lymphoid development. The expression of Eph/ephrin has been studied extensively in T-lymphocytes and expression of some of the members of this family including EphA1, EphA2, EphA3, EphA4, EphA7, EphB2, EphB6, ephrinA1 ephrinA3, ephrinA5 and ephrinB1 has been reported in the thymus, both on thymic stroma and lymphoid cells suggesting a role in T-cell development [128-130]. Interestingly, there are no reports of defective T-lineage development in knockout mice, perhaps a result of there being multiple Eph receptors with over-lapping functions in the T-cell compartment.

As well as T-lymphocytes the expression of Eph/ephrin gene has been also reported in B-lymphocytes [75,131,132]. EphA7 and EphA4 transcripts were found in human foetal bone marrow pre-B and pro-B cells (pre/pro B-cells). EphA4 expression is found in both adult and foetal pro-B and pre-B lineage cells with high levels of expression in peripheral blood. The full length EphA7 transcript, however, was not found in mature foetal B-lineage and adult B-lineage cells. This suggests that EphA7 may be involved in expansion and /or differentiation of pre/pro B-cell but is lost on mature B-cells [132]. Further studies show that there are different types of EphA7 mRNA, one of which encodes the full length EphA7 and another splice variant encodes a truncated, soluble

protein that lacks the cytoplasmic domain. Studies by Dawson et al [112] showed that normal lymphocytes express and secrete the truncated form of EphA7 [112], which has been shown to have tumour suppressive effects in lymphoma [84].

### 1.3.2 Eph/ephrin in vascular development

In the context of haematopoiesis, the vascular system is crucial in development of blood cells within the bone marrow and also in the function and migration of mature haematopoietic cells. The vascular system arises from two distinct processes known as vasculogenesis and angiogenesis. Vasculogenesis is an early event in embryonic development involving mesodermal cells differentiation to form a vascular plexus in embryonic tissues and angiogenesis is a process in which new blood vessels are formed from existing blood vessels. Angiogenesis has a role both in early development and in the adult haematopoietic system, it also promotes sprouting of new blood vessels in embryonic and postnatal vasculature and it has been shown to be important in development and metastasis of solid tumours [133]. Some members of Eph/ephrin family have established roles in vasculogenesis and angiogenesis. The expression analysis of Eph/ephrin using real-time polymerase chain reaction (RT-PCR) has shown expression of EphB2, EphB3, EphB4, ephrinB1 and ephrinB2 in the yolk sac [134]. EphrinB1 expression has been detected on both arteries and veins while the high affinity ligand for EphB4, ephrinB2, is only detected on arteries and EphB4 expression is only detected on veins. Knockout ephrinB2 mice and some of the EphB2 and EphB3 double mutants mice have defects in embryonic vasculature and therefore these mice are embryonically lethal. EphB2 and ephrinB2 expression in mesenchyme adjacent to vessels and the vascular defects in EphB2/EphB3 double mutants indicate a requirement for Eph/ephrin signalling between endothelial cells and surrounding mesenchymal cells [134]. EphA2 has also been reported to have a role in angiogenesis and the expression of EphA2 and its ligand ephrinA1 has been reported in both human and mouse tumour vasculature [99].

### 1.3.3 Eph/ephrin in bone remodelling and formation

The bone marrow is the principal site of haematopoiesis in adult animals and requires both vascular and other stromal cell types to create the haematopoietic niche. Important amongst these are the osteoblasts and osteoclasts, which mediate bone formation and remodelling. Bone is constantly remodelled through resorption of

mineralized bone by osteoclast and formation of new bone by osteoblast. Coupling of bone resorption and formation is critical during normal bone remodelling and it is necessary for bone growth, any deregulation in this process will result in pathological bone disease [56,115].

Eph receptors and ephrin ligands are important in bone remodelling and homeostasis during this process. Eph/ephrin bidirectional signalling regulates differentiation and function of the bone cells. Real time PCR (RT-PCR) analysis of the Eph/ephrin showed mRNA expression of ephrin ligands, ephrinB1, ephrinB2 and ephrinA1, A2, A4 and A5, as well as Eph receptors including EphB2, B3, B4, B6, EphA2, EphA3, EphA4 and EphA7 receptors on osteoblastic and osteoclastic cells [135-137]. Expression of EphB4 is observed on the osteoblasts and forward signalling through EphB4 results in bone formation and reverse signalling through ephrinB2 inhibit bone resorption therefore ephrinB2/EphB4 act as coupling stimulator [135]. Expression of ephrinA2 was observed during early osteoclastogenesis and unlike the ephrinB2 it acts as coupling inhibitor as reverse signalling through ephrinA2 result osteoclastogenesis and EphA2 forward signalling into osteoblast inhibit osteoblastic bone formation and mineralization [136]. EphrinA2/EphA2 bidirectional signalling facilitates bone remodelling at initiation phase, forward signalling through EphA2 receptor on osteoblast inhibit osteoblastic differentiation and bone formation and reverse signalling into osteoclast through ephrinA2 promote osteoclast differentiation [115,136]. EphrinB1 full knockout mice are prenatally lethal and they have skeletal defects. Studies on disruption of ephrinB1 on collagen I producing cells result in reduce bone formation and skull defect and studies on ephrinB1 conditional knockout mice shows defect in osteoblastic mediated bone formation with no increase in osteoclastic bone resorption and this condition results in reduction in bone size and density [138,139].

The importance of Eph/ephrin has also been shown in various stem cell niches, including: neural, dental and intestinal stem cell compartments [140,141]. More recent studies show their involvement in bone homeostasis and mesenchymal stem cell (MSC) regulation. Arthur et al [141] showed increase in osteogenic differentiation upon ephrinB1 and/or ephrinB2 expression by MSC. They also showed that ephrinB1 activation promoted chondrogenic differentiation. Therefore, EphB/ephrinB interactions may be involved in recruitment, migration and differentiation of MSC

during bone repair [141]. Studies by Ting et al [123] showed that ephrinA signalling interact with stem/progenitor cells in the bone marrow niche as its signalling mediates the release of progenitor cells from haematopoietic niche [123].

Interestingly, Eph/ephrin interaction is also involved in bone malignancies and tumours. Osteocarcinoma is a malignant bone tumour in adolescence and microarray analysis studies show increased expression of EphA2, EphA4, ephrinB1 and ephrinA1 in osteosarcoma cells [142,143].

### 1.3.4 Eph/ephrin expression in leukaemia and other haematopoietic tumours

Both chronic and acute myeloid leukaemias are malignant diseases of the haematopoietic system, which in most cases are believed to arise through the abnormal proliferation of either uncommitted or partially committed HSCs [34]. The origin of other types of leukaemia such as promyelocytic leukaemia, pre-B acute lymphoblastic leukaemia (Pre-B ALL), T-ALL and CLL are more likely due to malignant transformation of more mature progenitor cells. Expressions of elements of the Eph/ephrin system have been detected on many types of human leukaemias. One of the best studied is EphA3, which was originally identified in the LK63 pre-B ALL cell line and further investigations revealed its expression in T-cell leukaemia cell lines such as Jurkat, JM and HSB-2 [103,144]. It has been shown that EphA3 can induce both adhesive and cell repulsive responses in different cell types [145]. In analysing ephrin induced cell adhesion in LK63 cells, a critical role was identified for protein phosphatase activity, which prevented EphA3 phosphorylation and hence maintained the Eph/ephrin adhesive bond and prevented initiation of the signalling mechanisms leading to cell repulsion [145]. In leukaemia, EphA3 is expressed at significantly higher levels compared to normal blood cells, elevated expression of EphA3 has been detected in a proportion of clinical samples including cases of lymphoid and myeloid leukaemias [103]. Elevated EphA3 expression has also been detected on other cancers such as lung cancer, melanoma and brain tumours [59,102,103,145], where expression was found to be absent or low in corresponding normal tissues [92,103,146]. Recent array based studies showed EphA3 as one of the genes with loss in copy number alteration (CNA) in the genome of acute myeloid leukaemia (AML) patients [147]. Further studies by Guan et al [148] showed copy number variation (CNV) of EphA3 to be associated with

various types haematological malignancies and therefore CNV of EphA3 could be used as a diagnostic indicator for different types of leukaemia [148]. Many cancers including leukaemia require multiple cooperative oncogene mutations for malignant cell transformation. Specific sets of genes synergically dis-regulated by cooperative oncogenes are known as cooperative response genes (CRGs), which regulate leukaemia stem cell (LSC) growth and survival. Studies by Ashton et al [149] where stem cells were retrovirally-transduced with two fusion genes found in human myeloid leukaemias, NUP98-HoxA9 and Bcr-Abl, have identified EphA3 as a common CRG. They showed that shRNA knockdown of EphA3 in leukemic stem cells reduce leukemic cell engraftment, that this gene may be responsible for leukaemia stem cell growth and survival in bone marrow microenvironment [149]. With the involvement of EphA3 in many different types of leukaemia a high affinity monoclonal antibody to EphA3 (cIIIA4) [103] has been fully humaneered by Kalabios and the resulting antibody, KB004, is now in phase I clinical trial in leukaemia and other haematological cancers [150].

As mentioned previously EphB4 was originally identified in human bone marrow $CD34^+$ cells and its expression has been reported in erythroid progenitor cells, however its ligand ephrinB2 is expressed in bone marrow stromal cells [125]. Co-expression of EphB4 and ephrinB2 is found in the yolk sac, which is the first site of haematopoiesis and vascular development during embryogenesis. EphB4/ephrinB2 expression has been shown in the majority of leukaemia and lymphoma cell lines although expression in clinical samples appears less prominent [118]. Antibodies to EphB4 have undergone extensive pre-clinical evaluation and shown good anti-tumour effects in solid tumours which over-express EphB4 by inhibition of angiogenesis, however no efficacy has been shown for their effect in haematopoietic tumours to date [151]. Nevertheless, these antibodies may have the potential to be developed to target EphB4 in leukaemia and related blood cancers.

Studies by Nakanishi et al [113] shows EphA7 up regulation in the ALL1 associated leukaemia (ALL1/AF4 and ALL1/AF9). They also showed that EphA7 up-regulation was associated with phosphorylation of ERK and treatment with a phosphorylated ERK blocking drug resulted in apoptotic cell death in ALL1/AF4 leukemic blast cells [113]. Thus, anti-EphA7 antibodies or other inhibitors may well have a role in

leukaemia associated with this translocation. In contra-distinction to this positive role in leukaemia, EphA7 is lost in lymphomas, where the gene is hypermethylated and repressed in germinal center B-cell non-Hodgkins lymphomas and this has a potential to influence tumour progression and spread [112]. Further studies show EphA7 as targeted tumour suppressor gene in T-cell lymphoblastic lymphoma (T-LBL) and follicular B-cell lymphoma [84,152].

EphB6 expression has been observed in normal human tissue and over-expression of EphB6 has also been reported in both myeloid [153] and lymphoid leukaemias [119,154]. The expression level of EphB6 decreases with maturation of the cells in T-cell derived leukaemia-cells, therefore suggesting that EphB6 expression regulates T-cell development but has less significant role in mature T cells [119]. To date, there are no reports of experimental therapies targeting EphB6. Table 1.2 represent the summary of Eph receptors used as therapy target in various malignancies.

**Table 1.2. Eph receptors as a therapy target for cancer.**

| Eph | Targeted therapy | References |
|---|---|---|
| EphA1 | Tumour suppressor in colorectal cancer | 81 |
| EphA2 | EphA2 targeting reagents in ovarian cancer therapy | 155 |
| EphA3 | Therapeutic target in leukaemia and glioblastoma | 59,146 |
| EphA7 | Tumour suppressor in T-LBL and follicular lymphoma | 84,156 |
| EphB4 | EphB4 antibody to inhibit solid tumour growth | 151 |

In summary, the aberrant expression of Eph receptors in haematopoietic tumours reflects the spectrum of functions of these receptors in all cancers. In some cancers these genes act as tumour suppressor, examples being EphA1 in colorectal cancer and EphA7 in follicular lymphomas. On the other hand, these proteins can also have oncogenic effects, examples being the expression of EphA2 and EphA3 in glioma and the over-expression of EphA3 in leukaemia. In terms of therapy, the over-expression in certain tumours, taken together with the surface expression of these proteins, makes a strong case for targeted therapies. This is particularly the case when expression on normal tissues is minimal. This is exemplified by EphA3 and EphB4 where no toxicity was evident in pre-clinical testing of potential therapeutic antibodies. These studies reveal the therapeutic potential of targeting components of the Eph/ephrin

system in leukaemia and other cancers. These results should prompt further research into the specific roles of these proteins in different cancers as a prelude to designing and optimizing the therapeutic targeting of these proteins.

## 1.4 Hypothesis and Aims

### 1.4.1 Hypothesis

The available literature indicates that many leukaemias express high levels of Eph receptors but that any individual receptor is only expressed in a proportion of cases. The expression of the Eph/ephrin gene has been found in HSCs and leukaemia, however the function of this family of RTKs is not yet fully understood. Based on these preliminary data, I have hypothesized that the Eph genes have a role in the pathogenesis of leukaemia and in regulation of normal and leukemic haematopoietic stem cells and progenitor cells. It is envisaged that Eph proteins may provide novel therapeutic targets in leukaemia. To explore these hypotheses I have proposed the following aims:

**Aim 1** – To explore the expression and role of EphA receptors particularly EphA1, EphA2 and EphA7 in normal haematopoiesis.

**Aim 2** – To use animal models of acute leukaemia to explore the role of EphA2 genes by utilising EphA2 knockout mice and test for modification of leukaemogenesis and to test EphA2 targeted therapy.

**Aim 3** – To use EphA3 targeting agents (monoclonal antibodies and soluble inhibitors) as possible therapeutics in the murine xenograft and leukaemia models.

# Chapter 2 . TO EXPLORE THE SPECIFIC FUNCTION OF EPH GENES IN NORMAL HAEMATOPOIETIC CELLS

## 2.1 Introduction

Eph/ephrins belong to the largest family of receptor tyrosine kinases (RTKs). Eph signalling controls many cellular responses including cell adhesion, migration and invasion during development and homeostasis. Many members of this family of RTKs are strongly expressed at different stages of embryonic development and later in adult tissues. Severe embryonic defects in cardiovascular, skeletal or neural systems are observed upon deletion of some members of Eph/ephrin family [157]. Eph/ephrin together with fibronectin and integrin are involved in mesenchymal development and the signalling controls formation of tissue boundaries and neuronal growth. They also have a role in vasculogenesis and angiogenesis early in development. EphA2 expression has been reported in angiogenesis [99] and RT-PCR analysis showed expression of EphB2, EphB3, EphB4, ephrinB1 and ephrinB2 in the yolk sac [134]. Eph/ephrin signalling regulates cell movement and positioning in many developmental processes through control of cell adhesion and de-adhesion. These processes are present throughout life in the haematopoietic system. Thus, Eph/ephrin regulation, via cell-cell signalling, could provide a highly localised mechanism of regulation in the temporal-spatial development of haematopoietic cells.

Expressions of members of the Eph/ephrin family have been reported on mouse thymus and the expression is involved with T-cell development [64]. In situ hybridisation and RT-PCR analysis of thymus showed expression of Eph receptors on different thymocytes compartments, expression of EphA1 and EphA2 is detected on medullary stromal cells, EphA4 on thymic medulla and EphA7 on thymic cortex and subcapsule [111]. EphA1 expression has been detected on thymic medulla. Studies by Aasheim et al. showed expression of EphA1 and EphA4 on $CD4^+$ T-lymphocytes and suggested that activation of EphA1 through ephrinA1 ligand causes intracellular tyrosine phosphorylation, and actin polymerization, which result in an increased chemotactic response [158]. EphB6 is also expressed on $CD4^+$/$CD8^+$ T-cells, however it was shown that T-cell population in the EphB6 null mice is normal and these mice have no defect in T-cell development [130].

The expression of Eph/ephrin has been described on both malignant and normal B-cells. EphA3 was originally described on malignant pre B-cells [103] and EphA4 transcript is expressed on all B-cell populations obtained from foetal and adult bone

marrow [132]. Expression of full length EphA7 is detected on pre/pro B-cells but is lost on mature B-cells however expression of truncated form of EphA7 is found on both embryonic and mature B-cells, which suggests that EphA7 might be involved in expansion and differentiation of B-cells [112,132,159]. RT-PCR analysis showed mRNA expression of EphA2, EphA4, EphA7, EphB1 and EphB3 on dendritic cells obtained from $CD34^+$ progenitors [160]. EphB4 and ephrinB2 expression has been reported on erythroid precursors and the expression of EphA4, EphB1 and ephrinB1 has been detected on platelets [124,161].

To fully understand the effect of Eph genes on haematopoiesis, I will analyse the differentiated $lineage^+$ cells, progenitor cells and haematopoietic stem cells in EphA1, EphA2 and EphA7 knockout mice and their wild type littermates as these EphA receptors are widely expressed on many haematopoietic cells. Steidl et al. (2004) showed expression EphA1 along with its ligands ephrinA3 and ephrinA4 on human $CD34^+$ haematopoietic cells. Other studies showed expression of EphA1 on mouse HSCs, thymocytes and B-lymphocytes, which suggest a possible role for EphA1 in haematopoietic stem and progenitor cell positioning and function [121,123]. Expression of EphA2 has been reported on $LKS^+$ cells, thymocytes and dendritic cells and EphA2 has been reported to be involved with angiogenesis [123]. EphA7 is expressed on thymocytes and dendritic cells and is developmentally regulated during human B-lymphopoiesis, EphA7 expression has also been detected on mixed lineage leukaemia (MLL) [113,162]. Therefore, EphA1, EphA2 and EphA7 could be interesting targets for analysis in haematopoiesis.

EphA1 was the first member of Eph/ephrin family to be discovered and it was originally cloned from erythropoietin producing hepatocarcinoma cell line [38]. The highest affinity ligands for EphA1 are ephrinA1 followed by ephrinA3 and ephrinA4 with lower affinities, the expression of EphA1 along with its ligands is found during the early stages of development [163]. EphA1 expression is found on mouse epithelial tissues including skin, lung, liver, kidney, thymus uterus and vagina [88,164]. Like most other Ephs, over expression of EphA1 has been found on variety of cancers including skin, lung, liver, pancreas, breast, stomach and colon carcinomas [92]. EphA1 mutant mice were generated in our laboratory by insertion of an internal ribosome entry site (IRES)-human placental alkaline phosphatase (ALPP) reporter cassette into EphA1

exon-II gene to develop a genetic knockout. EphA1 is important in tissue pattering and the EphA1 knockout mice have two distinct phenotypes, the more common phenotype is development of kinky tail and the other one is imperforate uterovaginal development with hydrometrocolp, which is caused by resistance of cells to hormone-induced apoptosis [165].

EphA2 was first discovered in human epithelial carcinoma HeLa cells and was originally named epithelial cell kinase (Eck) for its expression in the majority of epithelial cells [166]. EphA2 has the ability to bind to all members of ephrinA ligands, however it could also function in a ligand independent manner [167,168]. EphA2 expression has been detected on embryonic tissues and is mainly found in the embryonic nervous system, embryonic spinal cord, in lens development and developing retina. EphA2 signalling is involved with regulation of cell growth, migration, and invasion and is strongly expressed in embryonic stem cells and during organogenesis. EphA2 is also found in adult tissues with low levels of expression on the surface of variety of proliferating epithelial cells including mammary glands, ovary, colon, kidney, and lung epithelial cells [167,168]. EphA2 is expressed in a range of different cancers including melanoma, breast, prostate and glioblastoma. EphA2 null mice were generated using the gene trap method, where most common phenotype of these mice is kinky short tail on a 129/Svj and C57BL/6 mixed background although this phenotype was lost when the mice where backcrossed with C57BL/6 mice [169].

Murine EphA7 was first discovered in central nervous system and human EphA7 was isolated from cDNA clones of human foetal brain library and was found to be expressed widely in human tissues [170,171]. Expression of EphA7 is found on various embryonic tissues including central nervous system and kidney vasculature [92,172]. Three forms of EphA7 have been discovered in human and mouse including one full-length EphA7 and two truncated forms of EphA7, where they lack an intracellular tyrosin kinase domain [173,159]. The expression is involved with different types of cancers including up-regulation in hepatocellular carcinomas and lung cancer and down-regulation in colon carcinomas [92]. The EphA7 knockout mice were generated using homologous recombination in the gene encoding EphA7 and by excising the first exon, which contained the start codon and signal peptide for the EphA7. EphA7 null mice were fertile and showed no morphological or behavioural defects [174].

As mentioned previously, expression of members of Eph/ephrin family of RTKs is found on many haematopoietic cells however currently there is a lack of knowledge in understanding the functional significances of the individual Eph/ephrins in haematopoiesis. Therefore, I have utilised EphA1, EphA2 and EphA7 knockout mice to investigate the effect of genetic deficiency of these Eph receptors on haematopoiesis. In this chapter the EphA1, EphA2 and EphA7 knockout mice were phenotyped and mature differentiated cells, progenitors and haematopoietic stem cells were analysed for haematopoietic abnormalities. This analysis will determine if individual Eph receptors have a significant role in normal haematopoiesis or alternatively if redundancy means loss of individual receptors will not be functionally significant.

## 2.2 Methods

### 2.2.1 Animals

Female mice of the congenic strains C57BL/6 (Ly 5.2) and PTPRC (Ly 5.1) were purchased from Animal Resource Centre (Perth, Australia) at the age of 5-6 weeks. C57BL/6 (Ly 5.2) and PTPRC (Ly 5.1) were crossed to produce double positive CD45.1/45.2 congenic mice. EphA1 knockout mice were developed in our laboratory and backcrossed to C57BL/6 for more than 10 generations [165]. EphA2 knockout mice were kindly supplied by Dr Naruse-nakajima (University of Tokyo)[169], these mice were similarly backcrossed with C57BL/6 (Jackson Labs) mice for 12 generations and subsequently maintained as homozygous knockouts on the C57BL/6 background. The EphA7 heterozygous mice were purchased from Jackson Laboratory, these mice were on a mixed background (129/C57BL/6) and were crossed with each other to develop homozygous mutant EphA7 mice and homozygous wild type EphA7 mice [175]. All of these mice were kept in QIMR Berghofer Medical Research Institute pathogen free animal facility according to institute protocols.

### 2.2.2 Immunophenotyping analysis

Peripheral blood was collected using submandibular blood sampling and analysed on a Hemavet analyser (Drew Scientific) to obtain the number of following blood parameters, hematocrit (HCT), white blood cell (WBC), red blood cell (RBC) neutrophil, lymphocyte, monocyte and platelet. For immunophenotyping analysis,

bone marrow and spleen tissues were collected from mice euthanized according to approved institute guidelines. To isolate bone marrow and spleen cells from these mice, bone marrow was flushed with a 26G needle and syringe containing 2% foetal bovine serum/phosphate-buffered saline (FBS/PBS) and filtered through 100 μm filters, spleen were dissociated, the cell suspension were washed through a 70 μm filter and red blood cells were lysed using red cell lysis buffer (Pharm Lyse™, BD Biosciences). All antibodies were obtained from eBioscience unless otherwise stated, and used at concentrations according to the manufacturer's instruction. For phenotyping of mature cells peripheral blood, bone marrow and spleen cells were stained with CD3 (12-0031-82) for T-cells, Gr1 (Biolegend, 108412) for Granulocytes and B220 (Biolegend 103204) for B-cells.

Bone marrow and spleen cells from EphA7 knockout mice were labelled for B-cell development and maturation analysis with the fluorescently conjugated antibody master mixes (B220, IgD and IgM) and (CD19, CD21 and CD23) provided by Professor Lynn Corcoran **(**Walter and Eliza Hall Institute of Medical Research) and SYTOX-Blue (Life Technologies) was used for separation of live cells. CD71 (12-0711-82) and Ter-119 (17-5921-82) antibodies were used for erythroid maturation analysis of bone marrow and spleen.

Total bone marrow cells from 6-8 week old EphA1, EphA2 and EphA7 knockout and wild type mice were counted on a Coulter Counter (BD Biosciences) machine. For analysis of haematopoietic stem and progenitor cells (HSPC) by flow cytometry, bone marrow cells from knockout and wild type mice were stained with a cocktail of biotinylated anti–mouse antibodies against antigens expressed on mature haematopoietic cells. Terminally differentiated cells (lineage) were labelled with a panel of biotinylated monoclonal antibodies to Ter119 (553672, BD Biosciences), CD3 (553060, BD Biosciences), CD5 (553019, BD Biosciences), B220 (553086, BD Biosciences), Mac-1 (553308, BD Biosciences) and Gr-1 (553125, BD Biosciences). The lineage$^+$ cells were detected with streptavidin (Biolegend, 405208) for phenotyping and were depleted with Dynabeads (Life Technologies) for sorting the HSCs. The lineage$^-$ cells were stained with a combination of C-kit (clone 2B8, 17-1171-82) and Sca-1 (clone D7, 25-5981-82). This population of cells contain two subpopulations known as Lineage$^-$c-Kit$^+$Sca-1$^+$ (LKS$^+$) cells and Lineage$^-$c-Kit$^+$Sca-1$^-$

progenitor cells. Progenitor cells were stained with CD34 (clone RAM34, 11-0341-82), FcγRII/III (clone 93, 12-0161-82) to further fractionate this population to GMP, CMP and MPP. $LKS^+$ cells were stained with either CD34 (clone RAM34, 11-0341-82) and Flk2 (clone A2F10.1, 12-1351-82) or CD150 (clone TC15-12F12.2, Biolegend 115912), and CD48 (clone HM48-1, Biolegend 103418) antibodies to further fractionate these cells into LT-HSCs, ST-HSCs and MPP cells. All flow cytometry data were analysed with FlowJo software (TreeStar).

### 2.2.3 Histological analysis

Whole spleen was dissected from EphA1, EphA2, EphA7 knockout mice and the corresponding wild type littermates and fixed in 10% formaldehyde followed by embedding in paraffin blocks and serial sectioning. The sections were stained with hematoxylin and eosin (H&E) and examined to determine pathology using Aperio XT (Leica Biosystems).

### 2.2.4 Competitive repopulation and transplantation assays

Bone marrow obtained from 6-8 week old EphA2 knockout or wild type control mice expressing the CD45.2 allotype antigen were flushed with 2% FBS/PBS and subjected to red blood cell lysis (Pharm Lyse™, BD Biosciences). For the competitive transplant experiment $1\times10^6$ viable, nucleated EphA2 knockout or wild type CD45.2 bone marrow cells were injected together with equal numbers of double positive CD45.1/45.2 congenic competitor bone marrow cells into the tail vein of lethally irradiated (11 Gy in 2 separate fractions at least 3 hours apart) CD45.1 recipient mice. Blood chimerism was analysed every 4 weeks for 16 weeks and on week 16 the bone marrow and spleen chimerism were determined. For secondary transplantation experiments, viable nucleated CD45.2 bone marrow cells from primary transplant were sorted on FACSAria (BD Biosciences) and injected, together with equal numbers of double positive CD45.1/45.2 congenic competitor bone marrow cells, into the lateral tail vein of lethally irradiated CD45.1 congenic secondary recipient mice and blood chimerism were analysed every 4 weeks for 24 weeks and on week 24 the bone marrow and spleen chimerism were determined. For determination of chimerism in transplantation assays, CD45.1 (clone A20, Biolegend 110708) and CD45.2 (clone 104, Biolegend 109814) antibodies were used in flow cytometric analysis and data were analysed with FlowJo software (TreeStar).

### 2.2.5 Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 6.01 software. Data are shown as mean ± SEM of at least three replicates, unless stated otherwise. Statistical significance was determined using an unpaired *t* test and a p-value $<0.05$ was considered significant.

## 2.3 Results

### 2.3.1 Analysis of full blood parameters, spleen weight, bone marrow and spleen nucleated cell numbers in EphA1, EphA2 and EphA7 knockout mice

To characterize haematopoietic function in EphA1, EphA2 and EphA7 knockout mice, I performed submandibular blood sampling on mice at 6-8 weeks of age. There were no statistically significant differences in baseline blood parameters, including hematocrit (HCT), white blood cell (WBC), red blood cell (RBC), neutrophil, lymphocyte, monocyte or platelet counts in any of the Eph knockout mice compared to wild type controls (Figure 2.1).

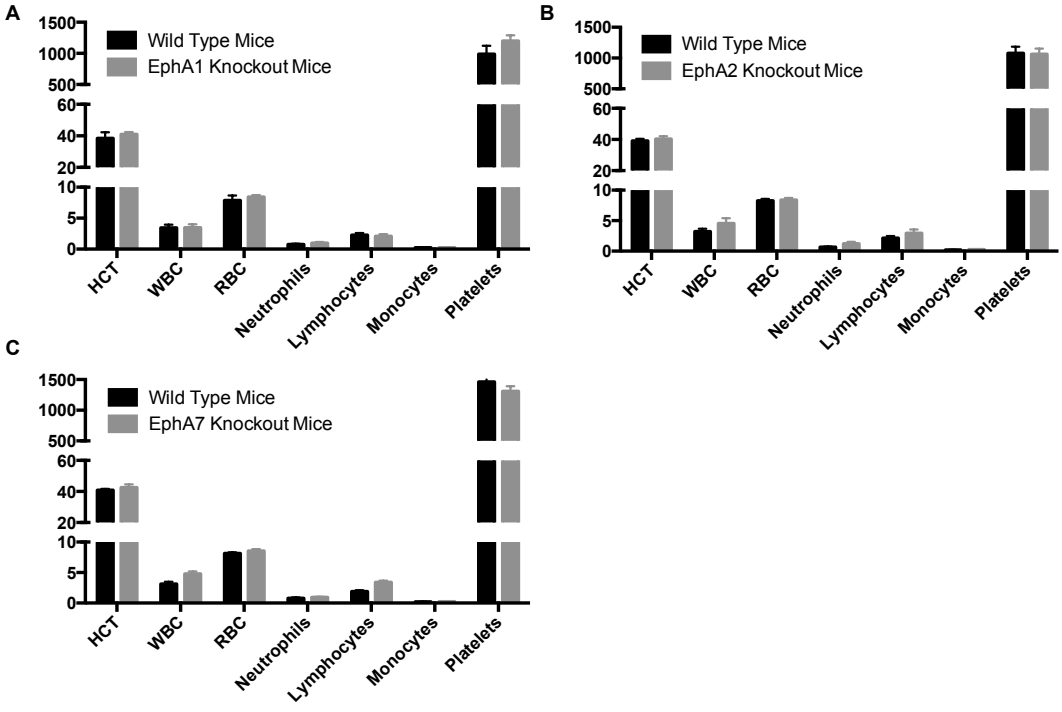



**Figure 2.1. Full blood count examination of EphA1, EphA2 and EphA7 knockout mice compared to wild type littermates.** (A) No significant differences in full blood differentiation of EphA1 knockout mice in comparison to EphA1 wild type mice were observed (n=10, 2 experiments). (B) No significant differences in full blood differentiation of EphA2 knockout mice in comparison to EphA2 wild type mice were observed (n=15, 3 experiments). (C) No significant differences in full blood differentiation of EphA7 knockout mice in comparison to EphA7 wild type mice were observed (n=10, 2 experiments). The data represent mean ± SEM. (n) represent the number mice used in each experiment. Unpaired *t* test was performed for statistical analyses. Each parameter is represented as HCT (HCT%), WBC ($x10^3/\mu L$), RBC ($x10^6/\mu L$), Neutrophils ($x10^3/\mu L$), Lymphocytes ($x10^3/\mu L$), Monocytes ($x10^3/\mu L$) and platelets ($x10^3/\mu L$).

The weight of spleen and liver in EphA1, EphA2 and EphA7 knockout mice was measured and compared to the wild type control. There were no significant changes observed in the size and weight of spleen and liver in any of the knockout mice compared to the wild type littermates. Number of nucleated bone marrow and spleen cells in EphA1, EphA2 and EphA7 knockout mice were compared to the wild type mice. The spleen and bone marrow showed no significant difference in number of nucleated cells (Figure 2.2).

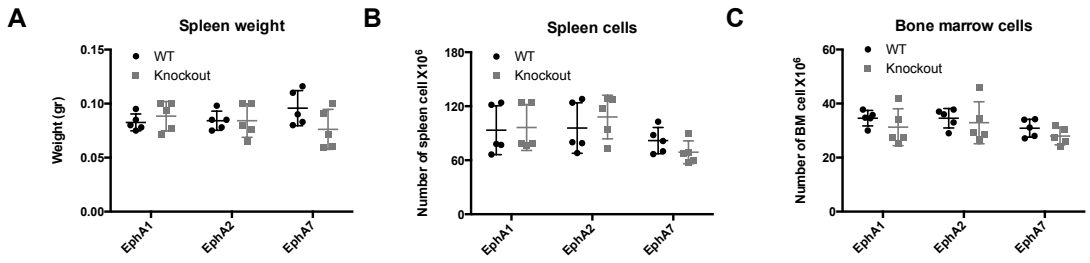



**Figure 2.2. EphA1, EphA2 and EphA7 knockout spleen weight and nucleated cell count compared to the wild type control.** (A) Spleen weight of EphA1, EphA2 and EphA7 knockout mice compared to the wild type littermates did not show any significant differences. (B) Number of nucleated spleen cells in EphA1, EphA2 and EphA7 knockout mice compared to the wild type littermates did not show any significant differences. (C) Number of nucleated bone marrow cells in EphA1, EphA2 and EphA7 knockout mice compared to the wild type littermates did not show any significant differences. Each dot corresponds to one individual mouse. The data represent the mean ± SEM. Unpaired *t* test was performed for statistical analyses.

### 2.3.2 EphA1, EphA2 and EphA7 bone marrow, spleen and blood immunophenotyping

Bone marrow, spleen and blood immunophenotyping analysis were used to identify the effect of lack of specific Eph expression on particular cell lineages. These analysis were used to identify differences between Eph knockout and wild type mice in mature blood cell populations including granulocyte (GR-1), B-lymphocyte (B220), or T-lymphocyte (CD-3) cell populations (Figure 2.3A).

Analysis of the lineage positive cells in EphA1 knockout mice showed significant reduction in bone marrow and spleen B-lymphocytes compared to the wild type control. There were also an elevated number of T-lymphocytes in both bone marrow and spleen of the knockout mice compared to the wild type control and a significant reduction in T-cell population in the blood of the EphA1 knockout mice. There were no differences between EphA1 knockout and wild type granulocyte populations (Figure 2.3B). Analyses of the granulocyte, B-lymphocyte and T-lymphocyte cell populations showed no significant differences in frequency of these cells in the EphA2 knockout mice compared to the wild type control mice (Figure 2.3C). In EphA7 knockout mice there were no differences in granulocytes population however a significant reduction in bone marrow T-lymphocytes and an increase in blood B-lymphocytes were observed (Figure 2.3D).

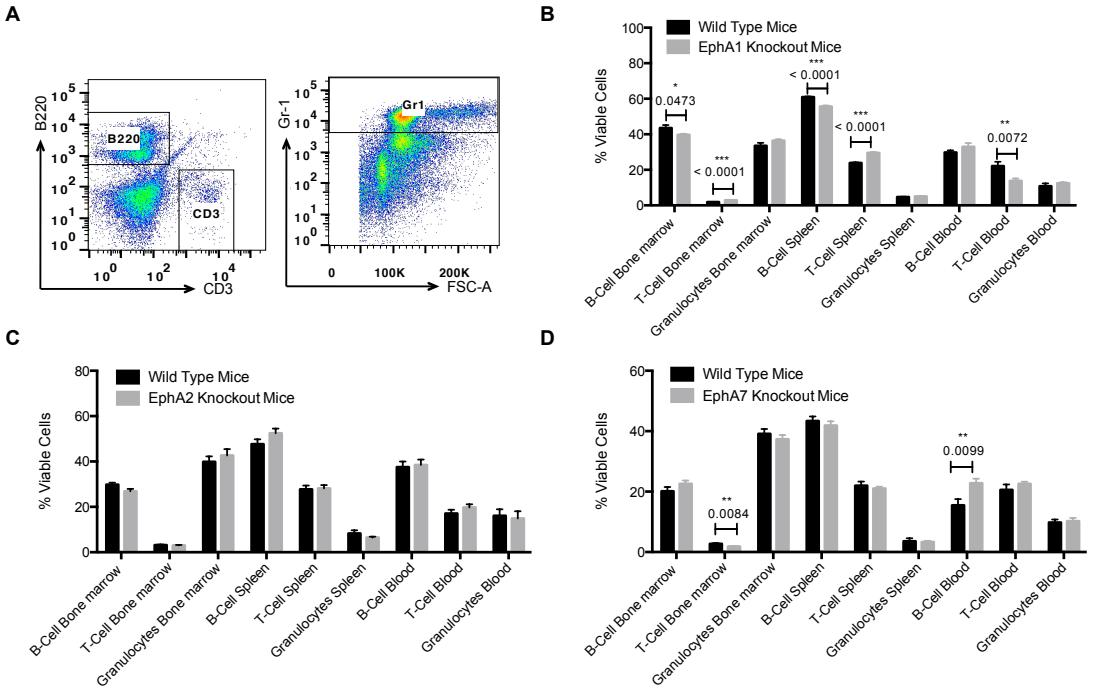



**Figure 2.3. B-lymphocytes, T-lymphocytes and granulocytes population in EphA1, EphA2 and EphA7 knockout mice.** (A) B-lymphocytes (B220), T-lymphocytes (CD3) and Granulocytes (Gr-1) gating strategy. (B) EphA1 knockout mice had significantly less B-lymphocytes in bone marrow (P value. 0.0475) and spleen (P value. <0.0001) compared to wild type littermates. EphA1 knockout mice have significantly more T-lymphocytes in bone marrow (P value. <0.0001) and spleen (P value. <0.0001) and significantly less T-lymphocytes in blood (P value. 0.0072) compared to wild type control mice. There were no significant differences in bone marrow, spleen and blood granulocytes population (n=10, 2 experiments). (C) There were no difference in B-lymphocytes, T-lymphocytes or granulocytes population in bone marrow, spleen and blood of the EphA2 knockout mice compared to the wild type control mice (n=15, 3 experiments). (D) EphA7 knockout mice have significantly less T-lymphocytes in bone marrow (P value: 0.0084) with no significant differences in spleen or blood T-lymphocytes population. EphA7 knockout mice had significantly more B-lymphocytes in blood (P value: 0.009) compared to wild type EphA7 mice and no difference in bone marrow or spleen B-lymphocytes population. There were no significant differences in EphA7 knockout bone marrow, spleen and blood granulocytes population compared to wild type mice (n=10, 2 experiments). Data are presented as percentage of viable bone marrow, spleen and blood cells. The data represent the mean ± SEM. (n) represent the number mice used in each experiment. An unpaired *t* test was performed for statistical analyses.

For evaluation of morphological changes in the EphA1, EphA2 and EphA7 knockout spleen, histological analysis of the knockout and wild type littermates were performed with documentation of any changes observed in the H&E sections. Spleen sections

from the knockout mice compared to wild type littermates displayed no changes in morphology and size of the spleen (Figure 2.4).

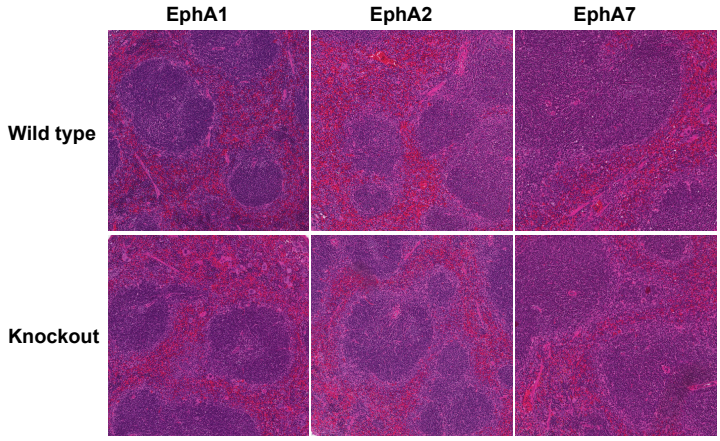



**Figure 2.4. Representative spleen sections from EphA1, EphA2, EphA7 and wild type littermates.** Spleen sections stained with H&E from EphA1, EphA2 and EphA7 knockout mice were phenotypically normal with no difference in morphology when compared to wild type littermates.

### 2.3.3 B-cell analysis in bone marrow and spleen of the EphA7 knockout mice

I sought to examine B lymphopoieis in EphA7 knockout mice based on the following factors: firstly, I have noted an elevated number of B-cells in blood of the EphA7 knockout mice and secondly, EphA7 has been shown to be a tumour suppressor in the context of B-cell lymphoma particularly in follicular lymphoma. This suggested that EphA7 mice might have an abnormality in B-cell lymphopoiesis due to a failure of selection mechanism in B-cell follicles. Therefore, I have analysed the successive stages of B-cell development in bone marrow and spleen cells from EphA7 mutant and wild type mice. In the bone marrow I have found that EphA7 knockout mice have no defect in pre/pro B-cells ($B220^{low}IgM^{-}$), transitional B-cells ($B220^{low}IgM^{+}$), and mature B-cells ($B220^{high}IgM^{+}$) (Figure 2.5A). Statistical analysis of the spleen $B220^{+}$ B-cells showed no significant difference in immature B-cells ($B220^{+}IgM^{+}IgD^{-}$) however there was a statistically significant increase in transitional B-cells ($B220^{+}IgM^{+}$ $IgD^{low}$) and a statistically significant decrease in mature B-cells ($B220^{+}IgM^{high}$ $IgD^{low}$) (Figure 2.5B). I further analysed the B-cell development in the spleen of the EphA7 knockout mice compared to the wild type littermates using CD19, CD21 and CD23 antibodies to discriminate between follicular B-cells ($CD19^{+}CD21^{int}CD23^{hi}$) and marginal zone B-cells ($CD19^{+}CD21^{hi}CD23^{lo}$). Total

splenic CD19$^{+}$ B-cells in the EphA7 knockout mice showed no significant differences in any of the marginal zone and follicular B-cell subpopulations (Figure 2.5C). Overall, there was no significant disruption in B lymphopoiesis despite some evidence for delayed maturation in the spleen.

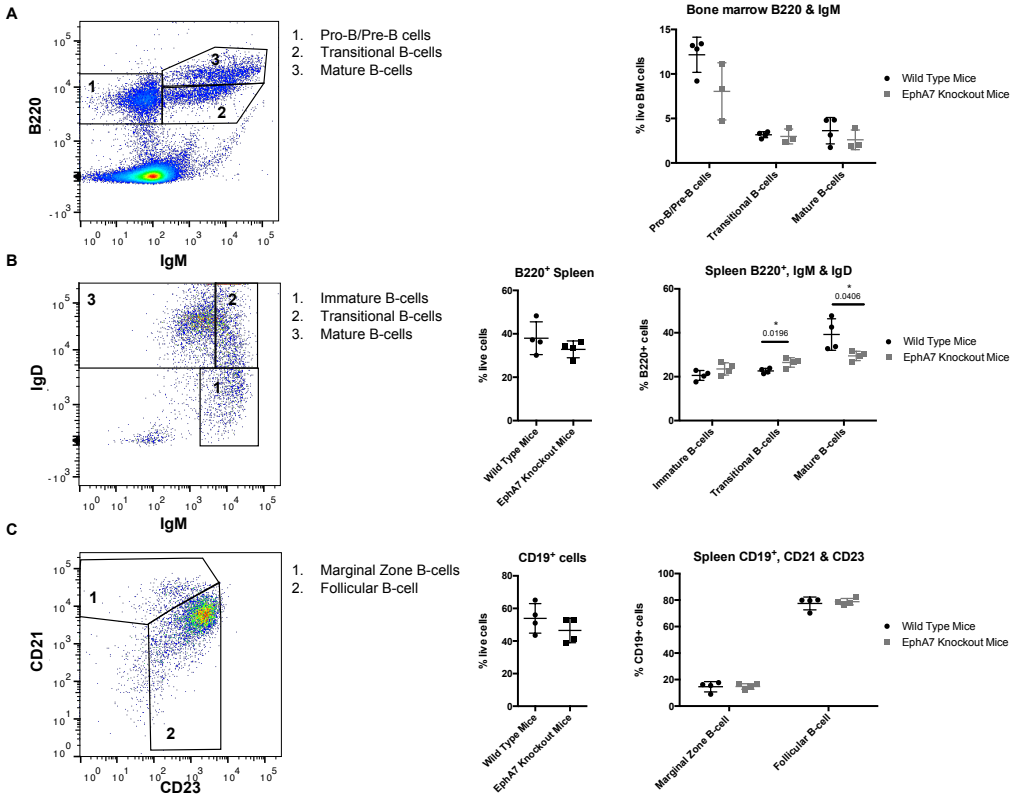



**Figure 2.5. Analysis of B-cell development in EphA7 knockout spleen and bone marrow cells.** (A) There were no significant differences between bone marrow population-1 pro–B/pre–B cells (B220$^{low}$IgM$^{-}$), population-2 transitional B-cells (B220$^{low}$IgM$^{+}$) and population-3 mature B-cells (B220$^{high}$IgM$^{+}$) obtained from EphA7 knockout mice compared to wild type mice (n=4). (B) In the spleen there were no significant differences in the B220$^{+}$ B-cell population. Total splenic B220$^{+}$ B-cells were gated and analysed for IgM and IgD expression. In the spleen B220$^{+}$ cells, there were no significant differences observed in population-1 immature B-cells (IgM$^{high}$IgD$^{low}$), there was a significant increase (P value. 0.0195) in population-2 transitional B-cells (IgM$^{high}$IgD$^{high}$) and a significant decrease (P value. 0.0406) in population-3 mature B-cells (IgM$^{low}$ IgD$^{high}$) in the EphA7 knockout mice compared to wild type mice (n=4). (C) In the spleen there was no significant difference in the CD19$^{+}$ B-cell population. Total splenic CD19$^{+}$ B-cells were gated and analysed for CD21 and CD23 expression. In the spleen CD19$^{+}$ cells there were no significant differences in population-1 marginal zone B-cells (CD21$^{hi}$CD23$^{lo}$) and population-2 follicular B-cells (CD21$^{high}$CD23$^{high}$) within the EphA7 knockout mice compared to wild type mice. Data are presented as percentage of viable bone marrow, spleen and blood cells. The data represent the mean ± SEM. (n) represent the number mice used in each experiment. An unpaired *t* test was performed for statistical analyses.

### 2.3.4 EphA1, EphA2 and EphA7 knockout bone marrow and spleen erythroid maturation

The stages of erythroid maturation were defined as erythoid-1 (Ery-1) corresponding to pro-erythroblasts ($CD71^{high}$, $Ter119^{mid}$), erythoid-2 (Ery2) corresponding to basophilic erythroblasts ($CD71^{high}$, $Ter119^{high}$), erythoid-3 (Ery3) corresponding to late basophilic and polychromatophilic erythroblasts ($CD71^{mid}$, $Ter119^{high}$), and erythoid-4 (Ery4) corresponding to orthochromatophilic erythroblasts ($CD71^{low}$, $Ter119^{high}$) (Figure 2.6A). Analysis of EphA1 and EphA7 knockout bone marrow and spleen cells for erythroid maturation showed no perturbation of erythropoiesis in comparison to wild type mice (Figure 2.6B, D). In the EphA2 knockout mice there was no perturbation of erythropoiesis in bone marrow, however spleen showed an increase in pro-erythroblast population with no changes in other stages of erythroid maturation (Figure 2.6C).

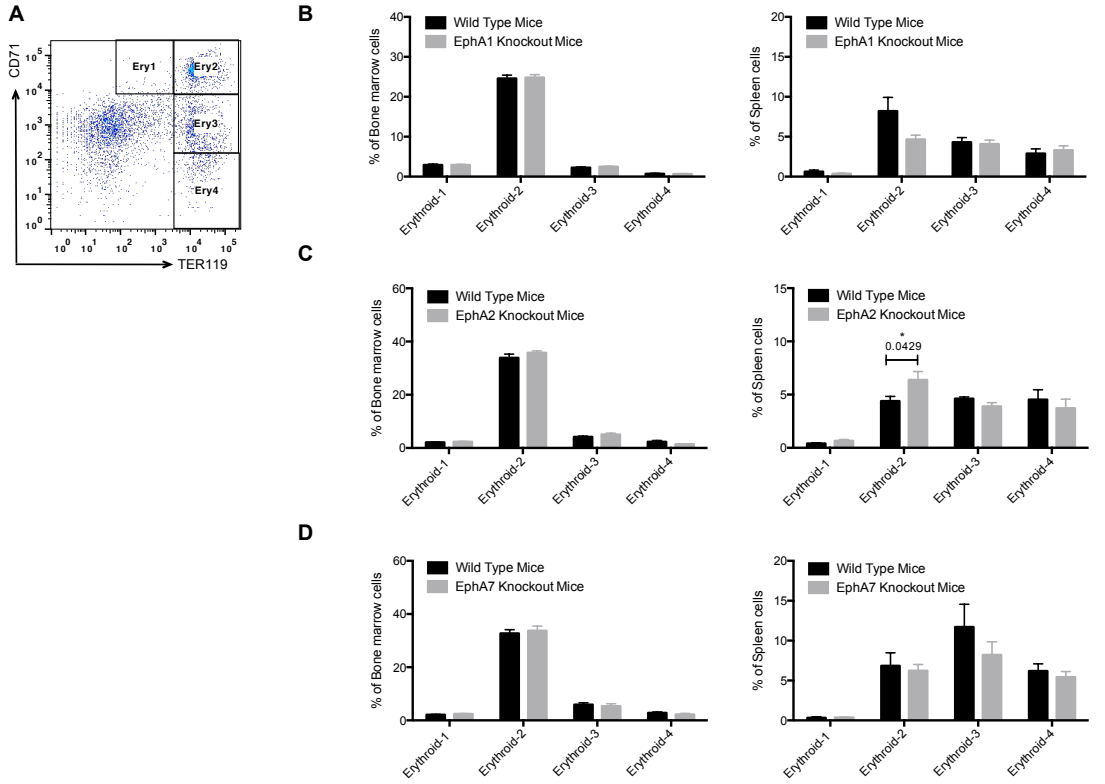



**Figure 2.6. Erythroid maturation analysis of EphA1, EphA2 and EphA7 knockout mice compared to wild type control mice.** (A) Ery-1 ($CD71^{high}$, $Ter119^{mid}$), Ery-2 ($CD71^{high}$, $Ter119^{high}$), Ery-3 ($CD71^{mid}$, $Ter119^{high}$) and Ery-4 ($CD71^{low}$, $Ter119^{high}$) gating strategy. (B) There were no differences in erythroid maturation in EphA1 knockout bone marrow or spleen cells compared to EphA1 wild type control cells (n=10, 2 experiments). (C) There were no differences in erythroid maturation of EphA2 knockout bone marrow cells compared to the wild type control cells. There was a significant increase (P value. 0.0429) in spleen Ery-2 population of the EphA2 knockout mice compared to the wild type control mice. There were no other significant differences at any stages of erythroid maturation in spleen cells compared to wild type control cells (n=15, 3 experiments). (D) There were no significant differences in erythroid maturation in EphA7 knockout bone marrow or spleen cells compared to EphA7 wild type cells (n=10, 2 experiments). The data is presented as percentage of bone marrow or spleen cells. The data represent the mean ± SEM. (n) represent the number mice used in each experiment. Unpaired *t* test was performed for statistical analyses.

### 2.3.5 Stem/progenitor cell populations in EphA1, EphA2 and EphA7 knockout mice

In this section the EphA1, EphA2 and EphA7 knockout haematopoietic stem and progenitor populations were compared to wild type littermates. I analysed the percentage of immunophenotypically defined haematopoietic stem and progenitor cells. This analysis showed no significant differences in the percentage of $LKS^{+}$ ($lineage^{-}Sca\text{-}1^{+}cKit^{high}Sca\text{-}1^{+}$), progenitors ($lineage^{-}Sca\text{-}1^{-}cKit^{high}$) and mature

myeloid progenitor cells, including granulocyte-monocyte progenitors (GMPs), common myeloid progenitors (CMPs), and megakaryocyte-erythrocyte progenitors (MEPs) (Figure 2.7).

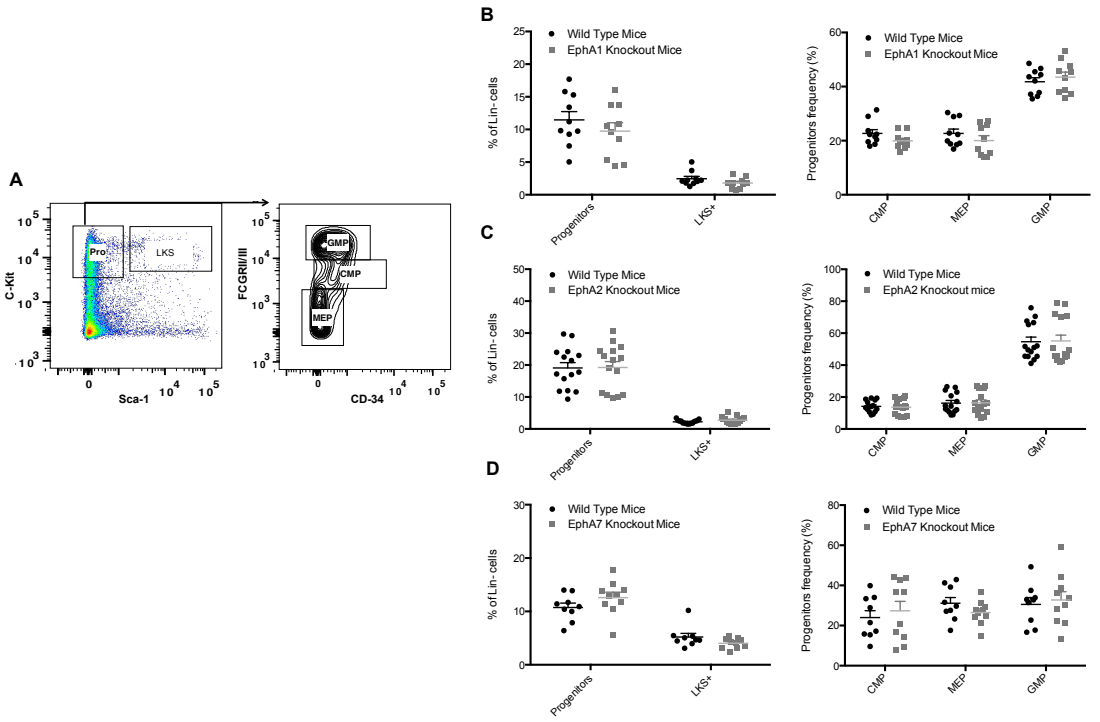



**Figure 2.7. LKS$^+$ and progenitor cell population in bone marrow of the EphA1, EphA2 and EphA7 knockout mice compared to wild type control.** (A) Gating strategy for LKS$^+$ cells (lineage$^{low}$cKit$^{high}$Sca-1$^+$) this population is enriched for haematopoietic stem cells, Progenitors (lineage$^{low}$cKit$^{high}$Sca-1$^-$), GMP (lineage$^{low}$cKit$^{high}$Sca-1$^-$CD34$^+$FCGRII/III$^{high}$), CMP (lineage$^{low}$cKit$^{high}$Sca-1$^-$CD34$^+$FCGRII/III$^{low}$) and MEP (lineage$^{low}$cKit$^{high}$Sca-1$^-$CD34$^-$FCGRII/III$^-$). (B) There were no differences in LKS$^+$, progenitors, CMP, MEP or GMP populations in EphA1 knockout mice compared to the wild type control mice (n=10, 2 experiments). (C) There were no significant differences in LKS$^+$, progenitors, CMP, MEP or GMP population in the EphA2 knockout mice compared to wild type control mice (n=15, 3 experiments). (D) There were no differences in LKS$^+$, progenitors, CMP, MEP and GMP population in EphA7 knockout mice compared to the wild type control mice (n=10, 2 experiments). Each dot corresponds to one individual mouse. The data presented as percentage of Lineage$^-$ bone marrow cells for progenitors and LKS$^+$ analysis. The data presented as frequency of progenitors for analysis of CMP, MEP and GMP population. The data represent mean ± SEM. Unpaired *t* test was performed for statistical analyses.

To further analyse HSCs, CD34 and FLK2 (CD135) markers were utilised to isolate LT-HSC (LKS$^+$CD34$^-$Flk2$^-$), ST-HSC (LKS$^+$CD34$^+$Flk2) or MPP (LKS$^+$CD34$^+$Flk2$^+$) populations (Figure 2.8A). No significant differences were observed in LT-HSC, ST-HSC or MPP populations in EphA1 or EphA7 knockout mice compared to the wild

type control (Figure 2.8B, D). EphA2 knockout mice showed a significant increase in the frequency of ST-HSC with no differences observed in the frequency of MPP or LT-HSC population (Figure 2.8C). CD150 and CD48 markers were also used as another method to fractionate the HSC, with ($LKS^{+}CD150^{+}CD48^{-}$) cell population representing LT-HSC and ($LKS^{+}CD150^{-}CD48^{+}$) representing MPP cells. Similar to the previous results, there were no significant differences in the frequency of LT-HSC or MPP cells in EphA1, EphA2 and EphA7 knockout mice compared to wild type control mice (Figure 2.8).

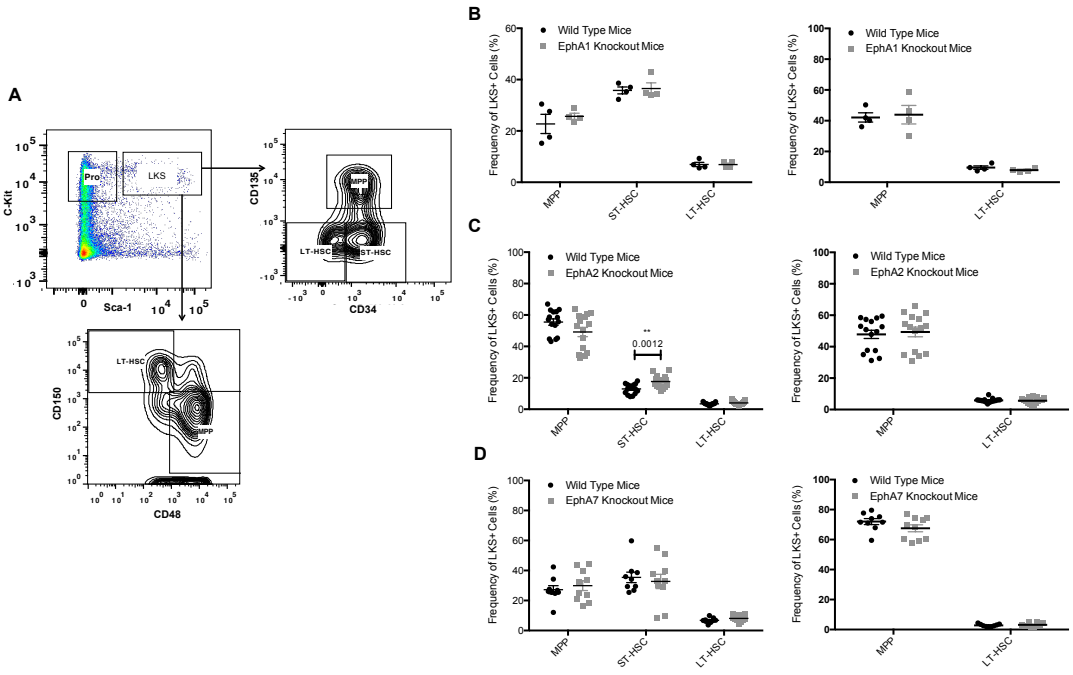



**Figure 2.8. Long term HSC (LT-HSC), short term HSC (ST-HSC) and multipotent progenitor population (MPP) in EphA1, EphA2 and EphA7 knockout mice compared to the wild type control mice.** (A) CD34/CD135 gating strategy, MPP ($LKS^+CD34^+CD135^+$), ST-HSC ($LKS^+CD34^+CD135^-$) and LT-HSC (LKS+CD34-CD135-). CD48/CD150 gating strategy, LT-HSC ($LKS^+CD150^+CD48^-$) and MPP ($LKS^+CD150^-CD48^+$). (B) There were no significant differences in LT-HSC, ST-HSC and MPP population gated by CD34/CD135 antigens in the EphA1 knockout mice compared to wild type controls. There were also no significant differences in LT-HSC and MPP population gated by CD48/CD150 antigens in the EphA1 knockout mice compared to wild type control (n= 4, 1 experiment). (C) There were no significant differences in LT-HSC and MPP population gated by CD34/CD135 antigens in the EphA2 knockout mice compared to wild type control however there were significantly more (P value. 0.0012) ST-HSCs in EphA2 knockout bone marrow compared to wild type control. There were no significant differences in LT-HSC and MPP population gated by CD48/CD150 antigens in EphA2 knockout mice compared to wild type control (n=15, 3 experiments). (D) There were no significant differences in LT-HSC, ST-HSC and MPP population gated by CD34/CD135 antigens in the EphA7 knockout mice compared to wild type control. There were also no significant differences in LT-HSC and MPP population gated by CD48/CD150 antigens in the EphA7 knockout mice compared to wild type control (n=10, 2 experiments). Each dot corresponds to one individual mouse. The data presented as frequency of LKS+. The data represent mean ± SEM. Unpaired t test was performed for statistical analyses.

### 2.3.6 EphA2 knockout bone marrow repopulating potential in primary and secondary transplant

EphA2 knockout mice showed an increase in the frequency of ST-HSCs which could indicate an altered stem cell dynamics, I aimed to explore whether there was functional difference in HSCs. Competitive bone marrow transplantation experiments were performed to assess the EphA2 knockout and wild type HSC self-renewal properties. No significant differences were observed in chimerism of EphA2 knockout at 4, 8, 12 and 16 weeks after transplantation (Figure 2.9A). Bone marrow and spleen chimerism analysis also didn't show any statistical difference between EphA2 knockout and wild type mice (Figure 2.9B). To assess whether there might be a more subtle effects of EphA2 knockout on long-term stem cell self-renewal in the murine haematopoietic compartment, a secondary competitive transplantation was performed. In the secondary transplant there were some mice with low percentage of chimerism, perhaps due to limiting numbers of LT-HSCs in the transplanted cell population. Importantly there were no significant differences between EphA2 knockout mice compared to the wild type control group (Figure 2.9C, D). This experiment was repeated with the same sized group of mice, and no significant differences were observed in either of these experiments indicating that lack of EphA2 does not have a significant functional effect on HSCs.

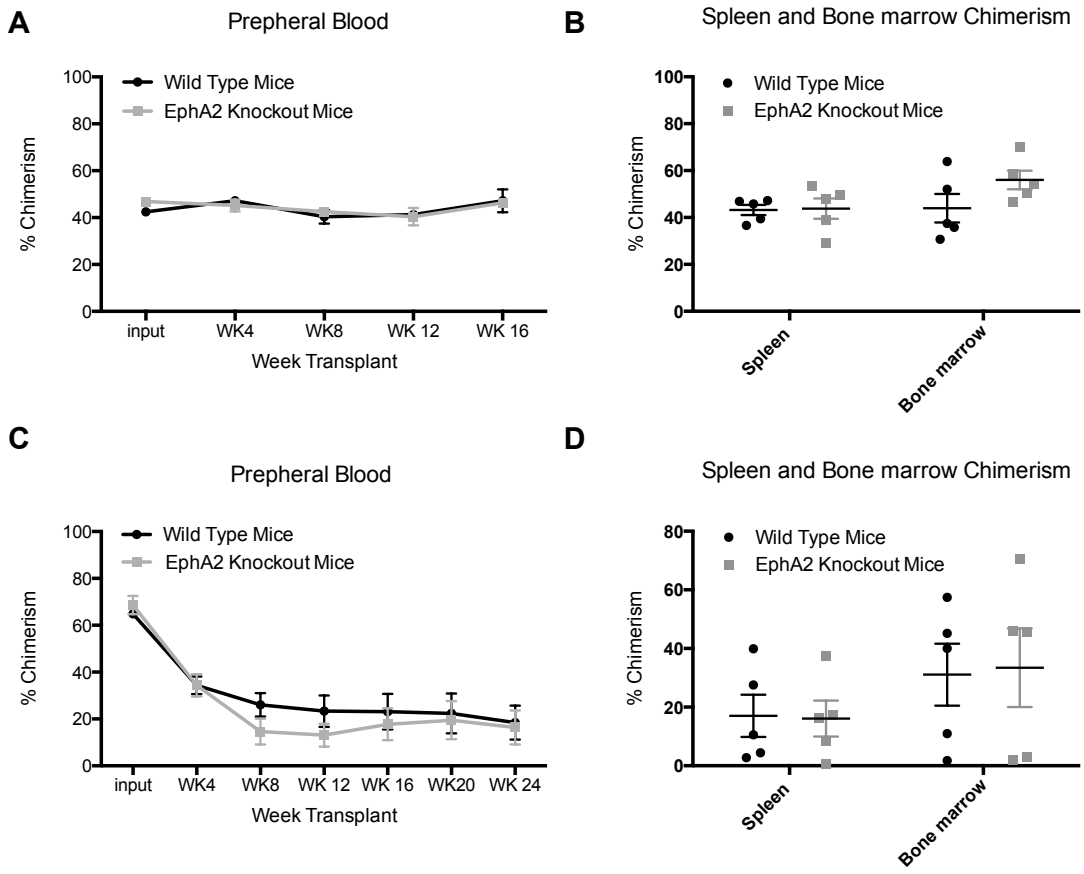



**Figure 2.9. EphA2 knockout bone marrow has normal repopulating potential in primary and secondary recipients.** (A) Whole-blood chimerism at 0, 4, 8, 12 and 16 weeks after transplantation of EphA2 knockout or wild type bone marrow cells into lethally irradiated CD45.1 recipients (n=5). (B) Analysis of bone marrow and spleen chimerism 16 weeks after primary transplantation. (C) Whole-blood chimerism of secondary transplant at 0, 4, 8, 12, 16, 20 and 24 weeks after transplantation of EphA2 knockout or wild type primary bone marrow cells into lethally irradiated CD45.1 recipient (n=5). (D) Bone marrow and spleen chimerism analysis 24 weeks after secondary transplantation. Each dot on panel A and C corresponds to mean and error from all wild type or knockout blood chimerism. Each dot on panel B and D corresponds to one individual mouse. The data presented as percentage of blood, bone marrow and spleen chimerism. The data represent mean ± SEM. An unpaired *t* test was performed for statistical analyses.

## 2.4 Discussion

Normal haematopoiesis requires complex bidirectional interactions between the bone marrow microenvironment (or niche) and haematopoietic stem cells (HSCs). These interactions are critical for the maintenance of normal HSCs quiescence and perturbations can influence HSCs self-renewal and differentiation. All lineages of blood are produced during the process of haematopoiesis and any disruption in this process can lead to different blood disorders. As mentioned previously, expression of members of Eph/ephrin family have been associated with different stages of blood development.

In this chapter, I have focused on analysis of haematopoiesis in EphA1, EphA2 and EphA7 knockout mice as the expression of these three receptors have been reported on many different haematopoietic cells and analysis of these knockout mice will facilitate understanding of the novel function of these receptors in haematopoiesis. Given the data that EphA3 is highly expressed on mouse HSCs and is found to be expressed in leukaemias [103,123], studies were planned for analysis of haematopoiesis in EphA3 knockout mice but profound delays in obtaining these mice precluded their use in my experiments.

Whole blood analysis showed no significant differences in blood parameters in any of the EphA1, EphA2 and EphA7 knockout mice compared to the wild type littermates.

I have also analysed the effect of these Eph receptors on differentiated lineage$^+$ cells in the knockout mice compared to the wild type littermates. EphA1 and EphA7 knockout mice had altered B-lymphocytes and T-lymphocytes populations compared to the wild type littermates. Unlike EphA1 and EphA7 knockout mice, EphA2 knockout mice did not exhibit any changes in T-lymphocytes and B-lymphocytes frequency. In addition, there was no disruption in bone marrow and spleen granulocyte frequencies in any of the EphA1, EphA2 or EphA7 knockout mice compared to wild type control.

My results showed a significant reduction in frequencies of B-lymphocytes in bone marrow and spleen of EphA1 knockout mice compared to the wild type littermates. Previous studies have shown expression of EphA1 on normal human B-cells isolated from peripheral blood or lymph node [154]. These data together with my results confirm

the possible effect of EphA1 on B-cell regulation. EphA1 is also expressed on peripheral blood T-lymphocytes and thymocytes and it has been suggested to have a role in regulation of $CD4^+$ T-cell entry into secondary lymphoid organs [158,176] thus suggesting a possible role of EphA1 in T-cell development and migration. My analysis of EphA1 knockout T-lymphocytes showed elevated number of T-cells in the bone marrow and spleen and a reduction in frequency of T-lymphocytes in blood of the EphA1 knockout mice. These results suggest that in the absence of EphA1, regulation of T-cells is disrupted hence an increase in spleen and bone marrow T-lymphocytes, and possibly the release of T-cells to the blood is blocked and as a result a decrease in frequency of blood T-lymphocytes was observed.

Expression of EphA7 has been documented on thymic cortex and subcapsule and the expression is involved with T-lymphocytes. EphA7 is also suggested as a tumour suppressor in T-cell lymphoblastic leukaemia/lymphoma due to its mutation in this type of cancer [177]. My results showed reduction in bone marrow T-cells in EphA7 knockout mice, which could be another indicator of the role of EphA7 in regulation of T-lymphocytes. I have also observed an elevated number of B-lymphocytes in blood of the EphA7 knockout mice, which could be an indication of a possible disruption in regulation of B-lymphocyte development and migration. Previous studies have shown possible involvement of EphA7 in B-cell regulation. It has been shown that mature B cells only express a splice variant encoding a soluble form of EphA7 [112,159,173]. A more recent study confirmed this finding and showed that EphA7 expression is lost in follicular lymphoma [84]. They went on to show that soluble EphA7 could bind to other Eph receptors, notably EphA2 and EphA3, thus preventing pro-oncogenic signalling by inhibiting clustering of membrane bound Eph receptors. The lack of soluble form EphA7 in the knockout mice may explain the increase in B-cell population, since the lack of inhibition of Eph signalling would lead to increased Erk and Src activity and hence may lead to increased proliferation. In this regard a study of B-cell subsets may reveal altered B-cell differentiation in the knockout mice. The studies performed by Orricchio [84] showing that EphA7 is a tumour suppressor suggests that crossing the EphA7 knockout mice with transgenic mice, such as the VavP-Bcl2 mice used in their study, might lead to more rapid lymphomagenesis.

Based on my finding and the previous studies on involvement of EphA7 in B-cell

lymphomas, I decided to look more closely at the stages of B-cell development in bone marrow and spleen of the EphA7 knockout mice compared to wild type littermates. B-cell development has been described in a linear process in which immature B-cells from bone marrow migrate to the spleen and give rise to transitional B-cells. These cells will then mature into marginal zone and follicular B-cells. The stages of B-cell maturation and development in the bone marrow of the knockout mice was not affected however the spleen showed an increase in transitional B-cells and reduction in the mature B-cells, indicating a delay in spleen B-cells maturation. Further analysis of B-cell development in the spleen did not show any significant disruption in marginal zone and follicular B-cells despite some evidence for delayed maturation in the spleen. This finding indicates that EphA7 is not functionally necessary for B-cell development in adult mice, although the functional redundancy between members of the Eph family of RTKs means that a role for Eph signalling cannot be excluded.

Erythroid maturation was also unaffected in EphA7 and EphA1 knockout spleen and bone marrow and EphA2 knockout bone marrow. There was an elevated number of spleen basophilic erythroblasts observed in EphA2 knockout spleen compared to wild type control however there were no significant difference in spleen late basophilic and polychromatophilic erythroblasts. Therefore, it can be concluded that lack of EphA2 may delay maturation but has no overall effect on erythropoiesis.

Previously Ting et al. (2011) [123] showed expression of many members of Eph/ephrin family on the haematopoietic stem and progenitor cells however my analysis of EphA1, EphA2 and EphA7 knockout mice showed no changes in the frequency of CMP, MEP or GMP progenitor cells suggesting that the differences seen in the differentiated B-lymphocytes and T-lymphocytes in EphA1 and EphA7 knockout mice are not caused by altered progenitor cells. There were also no differences in the frequency of LT-HSCs, ST-HSCs or MPPs in the EphA1 and EphA7 knockout mice, which suggest that these members of Eph family are not essential for haematopoietic stem cell function.

EphA2 knockout mice exhibited a significant increase in the number of ST-HSCs but no differences in the LT-HSCs and MPP populations, perhaps suggesting altered stem cell maturation. I therefore analysed the effect of EphA2 on haematopoietic stem cell

function. To determine overall stem cell self-renewal and maturation, I performed primary and secondary competitive transplantation assays. This was used to determine the repopulating ability of EphA2 knockout HSCs compared with congenic normal HSCs. The results indicate that EphA2-deficient and wild type bone marrow HSCs have the same contribution to haematopoiesis and the same capacity for self-renewal. Thus, despite the evidence that EphA2 is expressed on HSCs I have shown that loss of EphA2 does not alter HSC function.

Taken together, in this chapter I have shown that, although the expression of Eph/ephrin has been reported on many haematopoietic cells, loss of individual Eph genes results in, at most, only mild phenotypic aberrations of haematopoietic cells and that the EphA1, EphA2 and EphA7 knockout mice have essentially normal steady haematopoiesis. This does not exclude a role for the Eph family of RTKs overall, as there is considerable redundancy of function between related members of this family such that other Eph proteins might compensate for the absence of one member. Indeed, expression of multiple members of Eph family of RTKs has been reported on haematopoietic cells [75,121,123]. Therefore in haematopoiesis deletion of more than one Eph might reveal significant defects. For example, deletion of both EphA2 and EphA3, which are the highest expressing Ephs on mouse HSCs might have a more pronounced haematological defect [123]. Deletion of both EphA2 and EphA7 might be another interesting target for analysis of haematopoiesis as interaction of EphA2 and EphA7 in follicular lymphoma has been reported in which truncated form of EphA7 can bind to EphA2 receptor and act as inhibitor of EphA2 activation and block Erk and Src oncogenic signals [84]. Although beyond the scope of this thesis, another interesting avenue to explore would be to see if the haematopoietic system in these knockout models would show defects in the presence of acute stresses such as systemic infection or severe anaemia.

# Chapter 3 . THE ROLE OF EPHA2 IN ACUTE MYELOID LEUKEAMIA

## 3.1 Introduction

Leukaemia is blood cancer, caused by disruption in normal function of the haematopoietic system. This chapter will focus on analysis of specific Eph receptor in acute myeloid leukaemia (AML). AML is characterised by clonal proliferation of myeloid precursors and accumulation of immature haematopoietic cells in the bone marrow, blood and other tissues. This results in reduction in production of granulocytes, platelets and normal red blood cells (granulocytopenia, thrombocytopenia, and/or anaemia). Leukemic cells also cause direct pathology through hyperleukocytosis and organ infiltration [34,178].

The World Health Organization (WHO) classifies AML into four groups based on discrete biological entities and molecular pathways. The classification is based on a combination of morphology, immunophenotype, genetics, and clinical features and includes: AML with recurrent and characteristic genetic abnormalities which account for 11% of AML cases, AML with myelodysplasia related features which accounts for 6% AML cases, therapy related AML and MDS that accounts for 2% of AMLs and AML not otherwise specified which accounts for 81% of AML cases and includes AMLs that do not fall into the previous three groups [179].

AML is heterogeneous disease and has different responses to treatment based on factors including patient age, blast-cell morphology and cytogenetic abnormalities [180]. There are different types of treatments available for AML however depending on the sub-type of AML, these treatments, whilst often initially effective, do not prevent ultimate therapy-resistant relapse and death. Therefore, developments of new therapies, which target specific molecular mechanisms involved in the genesis of AML, have the potential to improve treatment outcomes [180].

AML with specific genetic abnormalities frequently have favourable prognoses with high rates of remission. Different genetic factors have been linked to this type of leukaemia with the most common targets being the genes that encode DNA-binding transcription factors or the genes involved in regulation of transcriptional complexes [181]. The most frequent mutations involve genes that regulate proliferation and survival of haematopoietic cells and those that control differentiation. The genetic abnormalities in this type of leukaemia includes: AML with t(8; 21)(q22; q22) showing the molecular abnormality in AML/ETO, AML with t(16; 16)(p13; q22) or

inv(16) showing the molecular abnormality in CBFβ/MYH11, acute promyelocytic leukaemia with t(15; 17)(q22; q12) leading to PML-RARalpha and AML with 11q23 alteration leading to mixed lineage leukaemia (MLL). MLL is associated with AML and ALL leukaemias and is a result of chromosomal translocations involving the MLL locus on chromosome 11. Some examples include: MLL-AF9 and MLL-AF6, which are consequence of chromosomal rearrangement in t(9;11) and t(6;11) respectively [162].

Many pathways and genes have been linked to MLL type leukaemias, including the glycogen synthase kinase 3 (GSK-3), heat shock protein 90 (HSP-90) and RAS pathways. Expression of many downstream mediators including clustered homeobox (HOX) proteins, myeloid ecotropic viral integration site 1 (MEIS1) and EphA7 have also been studied in MLL leukaemias [162]. Nakanishi et al [113] showed up regulation of EphA7 in MLL type leukaemias. They also showed that knocking-down EphA7 decreased ERK phosphorylation and treatment with an ERK inhibitor results in apoptotic cell death. Therefore, they suggested that EphA7 may have a role in leukaemia cell survival and it may be a potential therapy target for MLL type leukaemias. They have also detected expression of other EphA transcripts including EphA1, EphA2, EphA3, EphA4 and EphA6 in the MLL-AF9 and MLL-AF4 transfected K562 cells [113,162].

EphA2 is one of the most significantly expressed genes detected on MLL-AF9 transfected K562 cells. Expression of EphA2 has been detected in embryonic tissues, including embryonic nervous system and spinal cord. Low level of EphA2 expression is observed on adult epithelial tissues [168]. EphA2 is expressed on many different malignancies including breast, melanoma, ovarian, lung, glioma, prostate, cervical, colon and gastric cancers. EphA2 expression is involved in angiogenesis and tumour neovasculation and in some cancer the expression correlates with disease progression, the degree of malignancy and metastasis [67]. Studies by Brantley-Sieders on the implanted mammary adenocarcinoma in EphA2 deficient mice compared to the wild type mice showed decreased in the tumour volume, microvascular density and lung metastases. They also showed that EphA2 deficient endothelial cells have altered tumour vasculature and growth [182]. EphA2 expression is associated with poor prognosis and decrease survival; therefore EphA2 can be an inhibitor of tumour

progression and a very attractive therapeutic target in different types of cancers with EphA2 over-expression. Various therapeutic strategies targeting EphA2 expression in solid tumours have been developed including monoclonal antibody treatment, RNAi EphA2 suppression and EphA2 antibody combinational therapy with chemotherapeutic agents, radiotherapy and molecular therapies [183,184].

I have already shown that mice lacking EphA1, EphA2 and EphA7 have no significant defects in normal haematopoiesis. However, expression of EphA1, EphA2 and EphA7 was previously observed on MLL transfected K562 leukemic cells [113]. Therefore, the expression and functional effect of these Eph receptors in mixed-lineage leukaemia (MLL) will be analysed in this chapter utilising the MLL-AF9 mouse model of human AML leukaemia.

Given the role of Eph tyrosine kinases in signal transduction, and indeed reports of their interaction with the Abl/Arg tyrosine kinases [185]. I also chose to examine a model of leukaemia in which the kinome is disrupted through expression of the BCR-ABL fusion gene. In BCR-ABL chromosomal translocation occurs between chromosomes 9 and 22 resulting in formation of abnormal fusion proteins. The BCR-ABL fuses the Bcr and Abl kinase genes resulting in the formation of a unique Bcr-Abl protein in which the Abl kinase is constitutively active and is the causative molecular lesion of CML [36]. To develop this model of CML a construct containing the BCR-ABL transgene was used, which resulted in Abl kinase activation and development of leukaemia. Therefore this model of CML leukaemia was developed to determine if Eph expression might be present and having a part to play in pathogenesis of this type of leukaemia.

Firstly the expression of EphA1, EphA2 and EphA7 was examined in BCR-ABL and MLL-AF9 mouse models of human CML and AML leukaemias. In examination of Eph expression in these leukemic models I observed an elevated level of EphA2 and EphA7 transcript in MLL-AF9 leukaemia but no significant expression was observed in BCR-ABL leukemic cells and thus I focused on the MLL-AF9 model. Since reagents for EphA7 are still being developed, I analysed the effect of EphA2 in MLL-AF9 leukemic mice. EphA2 has a wide role in many different cancers and due to the existence of well-characterised antibodies I had the tools to assess whether it is a potential therapeutic agent in EphA2-positive MLL-AF9 leukaemias.

## 3.2 Methods

### 3.2.1 Animals

Female mice of the congenic strains C57BL/6 (Ly 5.2) were purchased from the Animal Resource Centre (Perth, Australia) at the age of 5-6 weeks. EphA2 knockout mice were kindly supplied by Dr Naruse-nakajima (University of Tokyo)[169]. EphA2 knockout mice were backcrossed with C57BL/6 (Jackson Labs) mice for 12 generations and maintained in the homozygous state. All of these mice were kept in QIMR Berghofer Medical Research Institute pathogen free animal facility according to institute protocols.

### 3.2.2 Mouse model of leukaemia

Retroviral supernatants were produced by transfection of 293T cells using FuGENE 6 reagent (Roche Diagnostics), retroviral vectors for packaging (Ecopac) and the retroviral constructs encoding GFP-control (pMSCV-IRES-GFP) for the control mice and either MLL-AF9 or BCR-ABL (pMSCV-MLL-AF9-IRES-GFP or pMSCV-BCR-ABL-IRES-GFP) for leukemic mice. Supernatants were collected 36–48 hours after transfection, passed through a 0.4 μm filter and concentrated to obtain supernatant with high titres of infectious retroviral particles. To generate the leukemic mice, wild type or EphA2 knockout cells from the femoral and tibial bone marrow of the donor mice were sorted on FACSAria (BD Biosciences) for HSC and cultured overnight on fibronectin in stem cell medium comprising of StemSpan (Stem Cell Technologies) serum-free medium supplemented with 100 ng/mL of Stem Cell Factor (SCF, PeproTech.), Thrombopoietin (TPO, PeproTech.), Granulocyte colony-stimulating factor (G-CSF) and Penicillin-Streptomycin (Life Technologies). The cultured cells were infected by incubation with retroviral supernatant (pMSCV-IRES-GFP, pMSCV-MLL-AF9-IRES-GFP, pMSCV-BCR-ABL-IRES-GFP) and 48 hours after infection viable, green fluorescent protein-positive ($GFP^+$) cells were sorted on FACSAria (BD Biosciences) for injection into sublethally irradiated (5.5 Gy) recipient mice. The mice were monitored for signs of leukaemia by analyses of whole blood including increase in WBC counts, $GFP^+$ cells, reduction in platelet and RBC count and for clinical evidence of disease including loss of weight, paucity of movement and hunching of the back. These mice were given a clinical score based on the symptoms including WBC count (0-2), percentage of $GFP^+$ cells (0-2), platelet

count (0-2), mouse weight (0-2) and movement (0-2) and culled once the clinical score reached 7 or more. Blood, spleen and bone marrow were harvested and processed into single cell suspensions for analysis and transplantation experiments. Bone marrow and spleen cells from the primary leukemic mice were sorted for viable $GFP^+$ cells on FACSAria (BD Biosciences). The secondary recipient mice (C57BL/6) were sublethally irradiated (5.5 Gy) and $2\times10^4$ EphA2 knockout or wild type MLL-AF9 leukemic cells from primary transplant were delivered via tail vein injection.

For assessment of the potential therapeutic effect of anti-EphA2 antibody on MLL-AF9 leukemic mice, the mice were treated intravenously with 0.1 mg of anti-mouse EphA2 monoclonal antibody (IF7 mAb) or vehicle only (PBS) control on alternate dates until the mice were euthanized due to progression of the disease.

### 3.2.3 Flow cytometric analysis

To examine cell surface expression of Eph proteins, $2\times10^6$ cells were washed in 2% foetal bovine serum/phosphate buffered saline (FBS/PBS). The cells were stained with 10 μg of anti-EphA1, -EphA2, -EphA3 and -EphA7 monoclonal antibodies or ephrinA5-Fc fusion protein directly conjugated to Alexa Fluor 647 or Alexa Fluor 488, and kept on ice for 30 minutes in the dark. Cells were washed twice with 2% FBS/PBS and stained with SYTOX-Blue (Life Technologies) dead cell marker and analysed on LSRFortessa (BD Biosciences).

### 3.2.4 Real-time quantitative polymerase chain reaction analysis

RNA was isolated from bone marrow of GFP-control, BCR-ABL and MLL-AF9 mice using RNeasy Mini Kit (QIAGEN) and complementary DNA (cDNA) was synthesized using SuperScript III (Life Technologies) with respect to the manufacturer's instructions. Relative transcript quantification was performed with SYBR Green QPCR Mastermix (Applied Biosystems) using RotorGene 3000 Real-Time PCR system gene expression was calculated relative to 18S rRNA or β-actin housekeeping gene.

### 3.2.5 Apoptosis analysis

Apoptosis was assessed using the Apoptosis Detection Kit (BD Biosciences) according to the manufacturer's instructions. Annexin V:Sytox Blue (Life Technologies) staining was assessed by flow cytometric analysis using LSRFortessa

(BD Biosciences).

### 3.2.6 Cell cycle analysis

Cell cycle was analysed on LSRFortessa (BD Biosciences), bone marrow and spleen cells were fixed and permeabilized using FIX & PERM® cell fixation and cell permeabilization kit (Life Technologies) and then stained with Hoechst 33342 (Life Technologies).

### 3.2.7 Radiolabelling and immunoreactivity

The EphA2 monoclonal antibody (IF7 mAb) and EphA3 monoclonal antibody (IIIA4 mAb) were conjugated to 1,4,7,10- tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid mono-(N-hydroxysuccinimidyl) (DOTA- NHS) ester as described previously [186]. Briefly, 2 mg/mL of IF7 or IIIA4 antibody solutions in 0.1 M sodium bicarbonate and 0.1 M sodium phosphate buffer (pH 8.5) were mixed with 50-fold molar excess of DOTA-NHS-Ester (Macrocyclics) dissolved in DMSO (Sigma Aldrich). The antibodies were conjugated for 1 hour at room temperature followed by fractionation to remove un-conjugated DOTA. The conjugate was buffer-exchanged into 0.2 M ammonium acetate (pH 5.5) using 100 kDa-cut-off microconcentrators as per the manufacturer's instructions (Millipore). For radiolabelling, purified DOTA-IF7 or DOTA-IIIA4 antibodies (4 mg/mL in ammonium acetate, pH 5.5) were mixed with Lutetium-177 (177 LuCl3, PerkinElmer) at final concentration of 10-12 mCi/mL for 1 hour at 37$^{o}$C. Radiolabelled immunoconjugate was purified using 100-kDa cut off microconcentrators according to the manufacturer's instructions. Specific radioactivity was determined using the ratio of Lutetium-177 radioactivity measured with AtomLab100 dose calibrator and the protein concentration measured using absorbance at 280 nm with NanoDrop 1000 spectrophotometer. The specific radioactivities were 8.1 and 8.5 mCi/mg (i.e. 300 and 316 MBq/mg) for $^{177}$Lu- IF7 and $^{177}$Lu-IIIA4 respectively. Treatments with PBS, unlabelled IF7, $^{177}$Lu-IF7 and $^{177}$Lu-IIIA4 antibodies were initiated 21 days after transplantation when the average percentage of GFP$^{+}$ cells in mouse blood reached 25% or more of viable blood cells. The treatments were administered intravenously via tail vein injection with two-dose administration of 10 μg with 3 MBq dose three days apart.

### 3.2.8 Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 6.01 software. Data are shown as mean ± SEM of at least three replicates, unless stated otherwise. Statistical significance was determined using an unpaired *t* test. The survival of the control and the treatment groups were compared using Kaplan–Meier analysis and statistical differences analysed using the log rank test. All flow cytometry data were analysed with FlowJo software (TreeStar).

## 3.3 Results

### 3.3.1 Expression of EphA1, EphA2 and EphA7 on leukemic xenograft models

Whilst I had shown that the expression of EphA1, EphA2 and EphA7 is not required for normal haematopoiesis, the increased expression of these genes in MLL transfected K562 cells raised the possibility that they may be functionally important in MLL type leukaemias [113]. I first examined the mRNA expression of these Ephs relative to 18S rRNA in bone marrow of GFP-control, BCR-ABL and MLL-AF9 leukemic mice using RT-PCR. Analysis of MLL-AF9 bone marrow from leukemic mice showed no significant increase in expression of EphA1 compared to GFP-control and BCR-ABL. However, the expression of EphA2 and EphA7 was significantly higher in bone marrow of MLL-AF9 leukemic mice compared to GFP-control (Figure 3.1A, C). Further analysis of EphA2 expression on MLL-AF9 mice using flow cytometry showed significantly higher EphA2 expression in bone marrow and spleen of MLL-AF9 compared to BCR-ABL and GFP-control mice (Figure 3.1D-F). Due to the lack of Eph expression in the BCR-ABL model this was not pursued further. However, elevated level of EphA2 expression in MLL-AF9 bone marrow and spleen raised the possibility that this protein may have a role in MLL-AF9 leukaemogenesis.

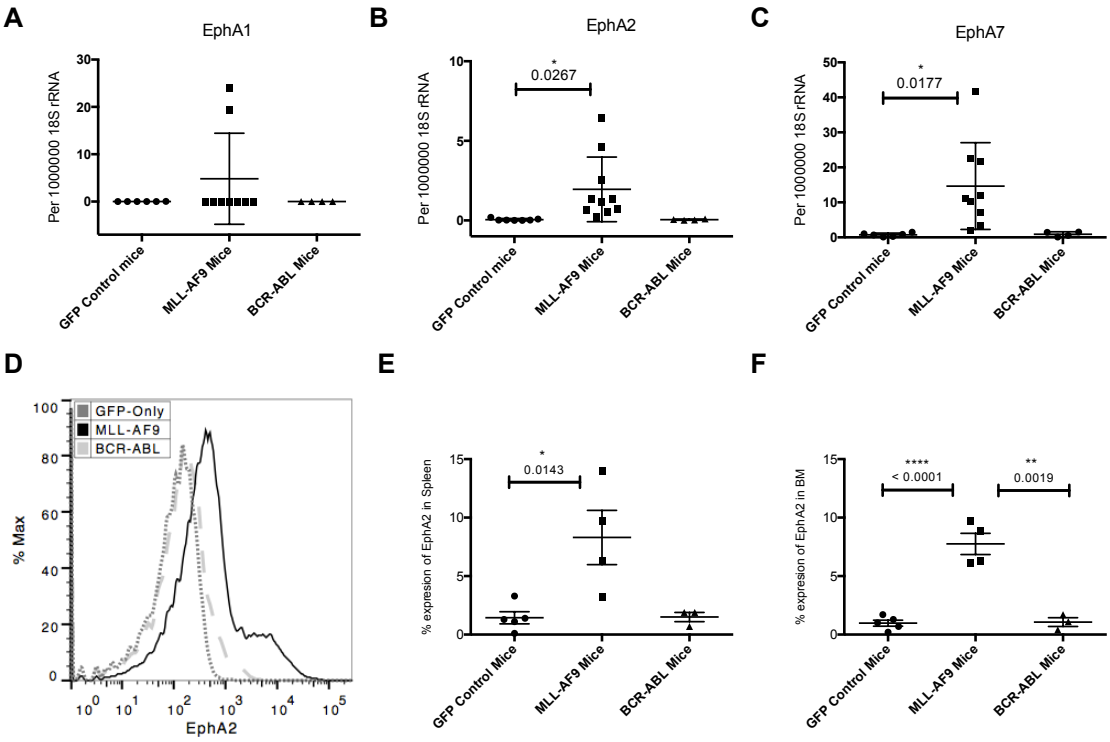



**Figure 3.1. EphA1, EphA2 and EphA7 expression on GFP-control, MLL-AF9 and BCR-ABL leukemic xenograft.** (A) The level of EphA1 mRNA expression relative to 18S rRNA from bone marrow of MLL-AF9 mice compared to GFP-control and BCR-ABL mice was not significantly different (n= 7 GFP-control, n=10 MLL-AF9, n=4 BCR-ABL). (B) The level of EphA2 mRNA expression relative to 18S rRNA showed significantly higher EphA2 transcript in bone marrow of MLL-AF9 mice compared to GFP-control (P value. 0.0267) and higher EphA2 transcript compared to BCR-ABL (n= 7 GFP-control, n=10 MLL-AF9, n=4 BCR-ABL). (C) The level of EphA7 mRNA expression relative to 18S rRNA showed significantly higher EphA7 transcript in bone marrow of MLL-AF9 mice compared to GFP-control (P value. 0.0177) and higher EphA7 transcript compared to BCR-ABL (n= 7 GFP-control, n=10 MLL-AF9, n=4 BCR-ABL). (D) Representative flow cytometric overlay of the EphA2 expression in GFP-control, MLL-AF9 and BCR-ABL bone marrow. (E) Flow cytometric analysis of the EphA2 expression on spleen of GFP-control, MLL-AF9 and BCR-ABL mice measured as mean fluorescent intensity, showed significantly higher EphA2 expression on spleen of the MLL-AF9 mice compared to GFP-control mice (P value. 0.0143) and higher EphA2 expression on MLL-AF9 compared to BCR-ABL leukemic spleen (n= 5 GFP-control, n=4 MLL-AF9, n=3 BCR-ABL). (F) Flow cytometric analysis of the EphA2 expression in bone marrow of GFP-control, MLL-AF9 and BCR-ABL mice is measured as mean fluorescent intensity, showed significantly higher EphA2 expression on MLL-AF9 bone marrow compared to GFP-control bone marrow (P value <0.0001) and higher expression of EphA2 on MLL-AF9 compared to BCR-ABL leukemic bone marrow (P value. 0.0019) (n= 5 GFP-control, n=4 MLL-AF9, n=3 BCR-ABL). Each dot corresponds to one individual mouse. The data represent the mean ± SEM. Unpaired *t* test was performed for statistical analyses.

### 3.3.2 The effect of EphA2 loss in MLL-AF9 induced mouse leukaemia

To explore the possibility that EphA2 was functionally important in MLL-AF9-driven leukaemias a series of MLL-AF9 leukemic transplantation experiments were performed (Figure 3.2A). Both C57BL/6 wild type and EphA2 knockout bone marrow cells were infected with the MLL-AF9 oncogene, the transduced bone marrow cells were transplanted into C57BL/6 wild type mice. The results from this transplantation showed that lack of EphA2 expression in the donor mice had no significant effect on leukemic engraftment (Figure 3.2B, C).

To test for a more subtle abnormality of leukemic stem cell (LSC) function in the absence of EphA2 in MLL-AF9 leukaemia, serial transplantations were set up to determine if LSC self-renewal in EphA2 knockout MLL-AF9 is different to the wild type MLL-AF9 LSCs. $2\times10^4$ $GFP^+$ leukemic cells harvested from primary transplanted MLL-AF9 mice were sorted on FACSAria for viable $GFP^+$ leukemic cells and injected into sublethally irradiated (5.5 Gy) C57BL/6 recipient (secondary transplant). The leukemic cells from both EphA2 knockout and wild type MLL-AF9 mice induced leukaemia with comparable disease latencies (Figure 3.2D). There were no significant differences in bone marrow, spleen and blood engraftment and in spleen and liver weight in EphA2 knockout and wild type MLL-AF9 mice at the time of cull in the secondary transplantation experiment (Figure 3.2E). The primary MLL-AF9 transplantation experiment was performed twice and the secondary MLL-AF9 transplantation experiment was set up four times with different primary donors. This result indicates that absence of EphA2 does not affect self-renewal of MLL-AF9 LSC in vivo.

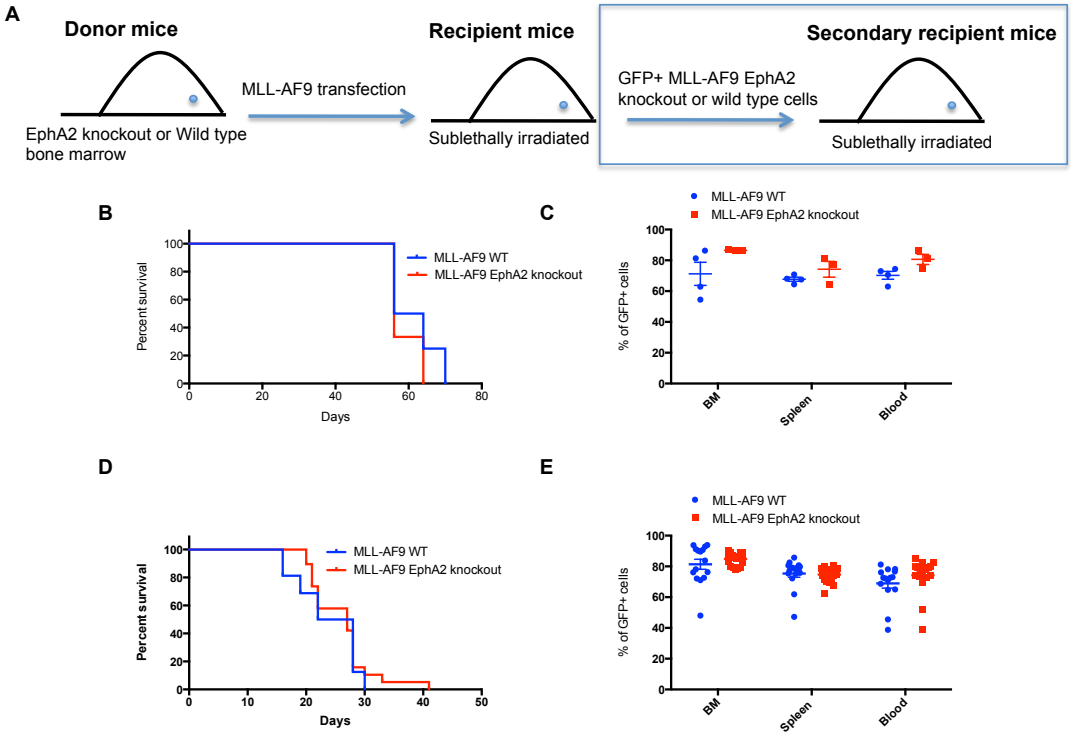
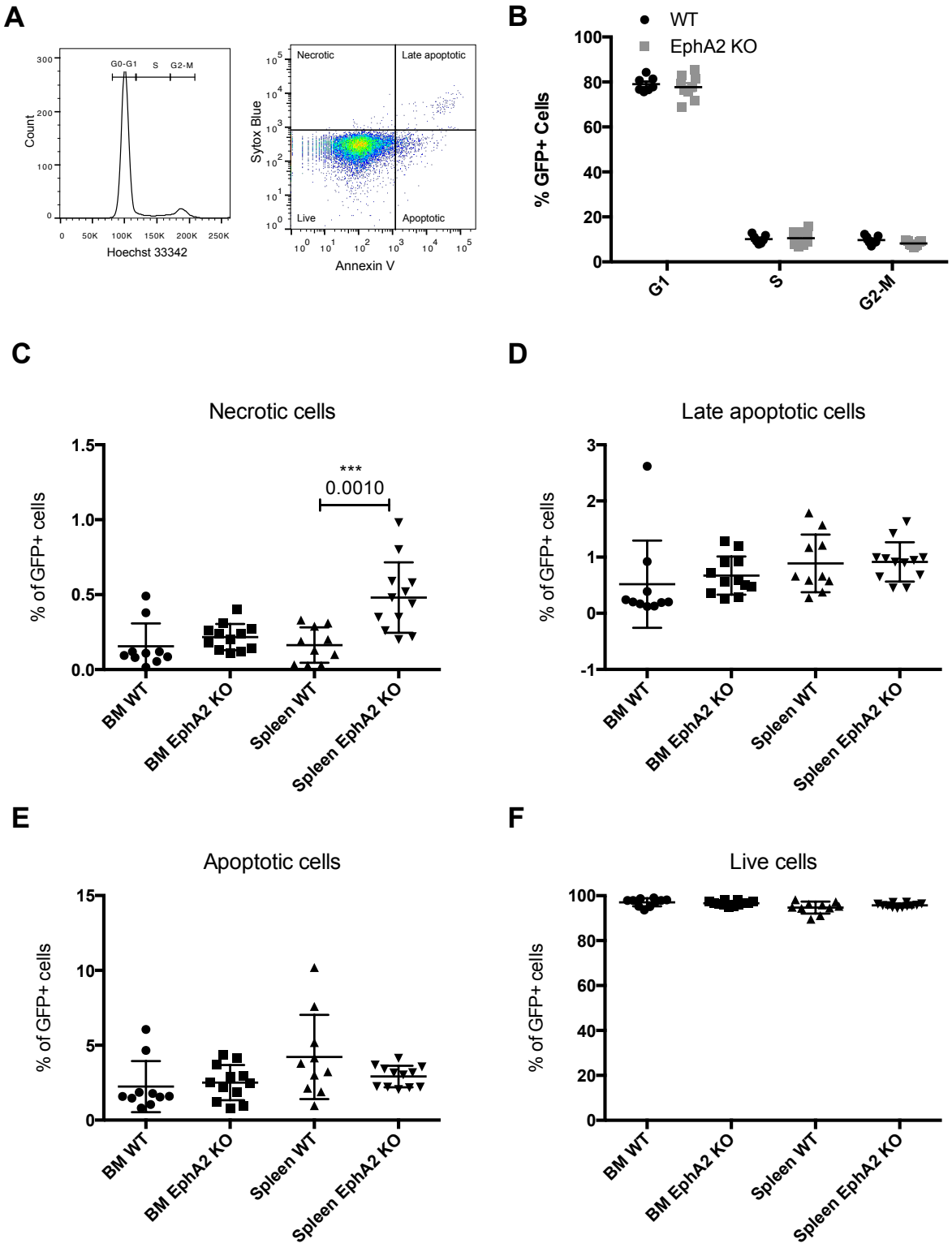



**Figure 3.2. MLL-AF9 EphA2 knockout mice have similar leukaemogenic potential as the wild type MLL-AF9 mice in primary and secondary leukemic transplant models.** (A) Serial MLL-AF9 leukemic transplantation model. (B) Survival of leukemic mice transplanted with EphA2 knockout MLL-AF9–transduced haematopoietic stem cells ($LKS^+$) compared to wild type MLL-AF9–transduced haematopoietic stem cells ($LKS^+$) showed no significant differences between the two groups (n= 4 wild type, n=3 EphA2 knockout, 2 experiments). (C) Percentage of $GFP^+$ cells in bone marrow, spleen and blood of the EphA2 knockout and wild type MLL-AF9 bone marrow, spleen and blood at time of cull didn't show any significant differences between the two groups. (D) Survival of secondary MLL-AF9 mice transplanted with $GFP^+$ EphA2 knockout or $GFP^+$ wild type MLL-AF9 cells from the primary transplant showed no significant differences between the two groups (n=16 wild type, n=19 EphA2 knockout, 4 experiments). (E) Percentage of $GFP^+$ cells in bone marrow, spleen and blood of the secondary transplanted wild type or EphA2 knockout MLL-AF9 mice didn't show any significant differences between the two groups. Each dot corresponds to one individual mouse. The survival is presented as Kaplan-Meier survival curve. The data represent the mean ± SEM. Unpaired *t* test was performed for statistical analyses.

To determine whether EphA2 loss had any effect on cell proliferation or survival in MLL-AF9 leukemic mice, cell cycle and apoptosis analysis were subsequently performed on $GFP^+$ MLL-AF9 bone marrow and spleen cells obtained at the time of cull from EphA2 knockout and wild type mice. Analysis of the bone marrow cells from EphA2 knockout and wild type leukemic mice using Hoechst 33342 showed no significant changes in any of the G1/S/G2-M fractions of the cell cycle profile (Figure

3.3B). Flow cytometric analysis of apoptosis in the spleen and bone marrow cells from EphA2 knockout and wild type MLL-AF9 mice using annexin V and SYTOX-Blue showed no significant difference in bone marrow apoptosis. However, the apoptosis analysis of the spleen cells obtained from the EphA2 knockout MLL-AF9 mice showed a significant increase in necrotic cells compared to wild type spleen cells from MLL-AF9 mice (Figure 3.3C-F). In summary, lack of EphA2 does not significantly alter cell cycle and apoptosis of the MLL-AF9 leukemic cells except for an increased observed in the percentage of spleen necrotic cells.


A
G0-G1
S
G2-M
Count
Hoechst 33342
Necrotic
Late apoptotic
Live
Apoptotic
Sytox Blue
Annexin V
B
WT
EphA2 KO
% GFP+ Cells
G1
S
G2-M
C
Necrotic cells
% of GFP+ cells
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0.0010
BM WT
BM EphA2 KO
Spleen WT
Spleen EphA2 KO
D
Late apoptotic cells
% of GFP+ cells
BM WT
BM EphA2 KO
Spleen WT
Spleen EphA2 KO
E
Apoptotic cells
% of GFP+ cells
BM WT
BM EphA2 KO
Spleen WT
Spleen EphA2 KO
F
Live cells
% of GFP+ cells
BM WT
BM EphA2 KO
Spleen WT
Spleen EphA2 KO

**Figure 3.3. Cell cycle and apoptosis analysis of the EphA2 knockout MLL-AF9 mice compared to wild type MLL-AF9 mice.** (A) Gating strategy for cell cycle and apoptosis analysis respectively. (B) Cell cycle analysis of the EphA2 knockout MLL-AF9 mice compared to the wild type MLL-AF9 mice showed no significant differences in any of G1/S/G2-M cell cycle fractions (n= 7 wild type, n=9 EphA2 Knockout, 2 experiments). (C) Percentage of necrotic cells in $GFP^+$ MLL-AF9 bone marrow was not significantly different in leukemic mice compared to wild type MLL-AF9 mice, however there were a significant increase (P value. 0.0010) in the percentage of $GFP^+$ necrotic spleen cells in EphA2 knockout MLL-AF9 spleen compared to wild type spleen cells. (D) Percentage of late-apoptotic cells in $GFP^+$ MLL-AF9 bone marrow and spleen cells was not significantly different between EphA2 knockout mice compared to wild type control mice. (E) Percentage of apoptotic cells in $GFP^+$ MLL-AF9 bone marrow and spleen cells was not significantly different between EphA2 knockout mice compared to wild type control mice. (F) Percentage of live cells in $GFP^+$ MLL-AF9 bone marrow and spleen cells was not significantly different between EphA2 knockout mice compared to wild type control mice (n=10 wild type, n=12 EphA2 Knockout, 2 experiments). Each dot corresponds to one individual mouse. The data represent the mean ± SEM. Unpaired *t* test was performed for statistical analyses.

### 3.3.3 Analysis of EphA expression in MLL leukaemia

A possible explanation for the lack of effect of EphA2 deletion in MLL-AF9 leukaemia is that other Eph proteins with overlapping ephrin-binding affinities, such as EphA7, might show a compensatory increase in expression. To explore this possibility I analysed EphA7 expression and also total EphA expression on EphA2 knockout and wild type MLL-AF9 leukemic cells using flow cytometric analysis. Total EphA expression was determined by binding of ephrinA5-Fc with high binding affinity for members of EphA subfamily. In EphA2 knockout MLL-AF9 leukemic cells, EphA7 expression was not significantly up-regulated compared to wild type MLL-AF9. However, the levels of ephrinA5-Fc binding in EphA2 knockout MLL-AF9 was not significantly different to wild type MLL-AF9 cells suggesting that, despite loss of EphA2, the complement of Eph receptors was similar on MLL-AF9 leukemic mice (Figure 3.4A). This supported the possibility that some compensatory increase in other Eph genes was present in the EphA2 knockout leukemic cells. Thus, I performed a RT-PCR analysis of the MLL-AF9 leukemic bone marrow cells from EphA2 knockout and wild type mice to determine the expression of other EphA genes. This again showed no significant change in EphA7, in support of the flow cytometric data. There was an increase in EphA5 expression in EphA2 knockout

MLL-A9 when compared to wild type MLL-AF9 leukemic cells but this did not reach statistical significance (Figure 3.4B).

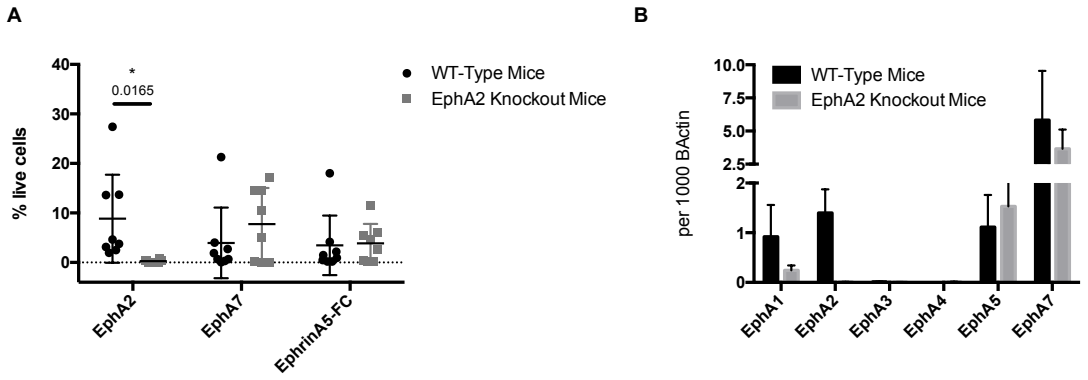
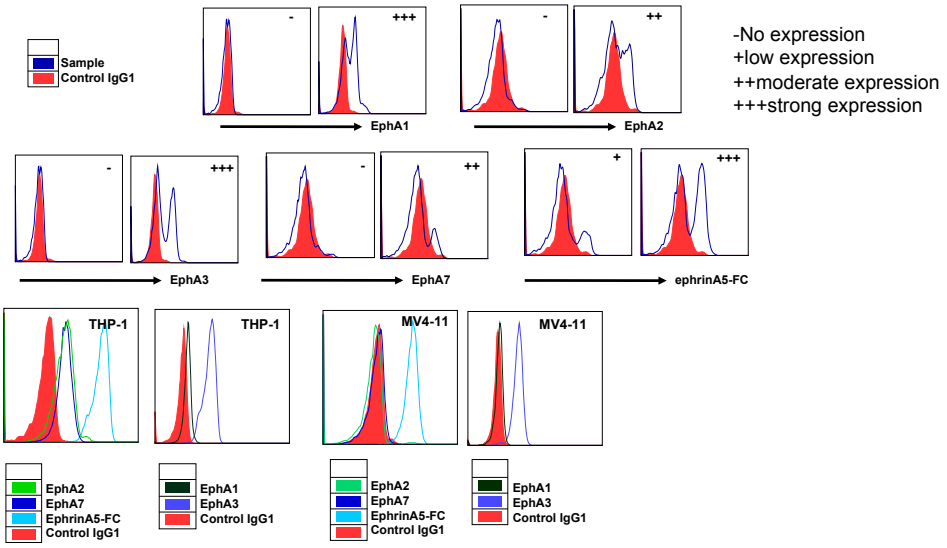



**Figure 3.4. Expression analysis of EphA subfamily on EphA2 knockout and wild type MLL-AF9 mice.** (A) Flow cytometric analysis of EphA2, EphA7 and ephrinA5-Fc in EphA2 knockout and wild type bone marrow cells showed no EphA2 expression in the EphA2 knockout mice and significantly high expression of EphA2 in the wild type mice as expected (P value. 0.0165). Comparable levels of EphA7 (P value. 0.3099) and ephrinA5-Fc (P value. 0.8710) expression were observed in EphA2 knockout and wild type mice. (B) RT-PCR analysis of EphA1, EphA2, EphA3, EphA4, EphA5 and EphA7 in EphA2 knockout and wild type bone marrow cells showed no EphA2 transcript in the EphA2 knockout mice compared to wild type MLL-AF9 (P value. 0.0776) and comparable level of EphA1 (P value. 0.3458), EphA5 (P value. 0.7020) and EphA7 (P value. 0.6167) transcript in EphA2 knockout and wild type mice. There were no expression of EphA3 and EphA4 observed in any of the MLL-AF9 mice (n=4). Each dot corresponds to one individual mouse. The data represent the mean ± SEM. Unpaired *t* test was performed for statistical analyses.

To further evaluate the expression of Eph family of RTKs on MLL type leukaemias. I have analysed eight clinical samples from MLL-driven leukaemias and two human cell lines (THP-1 and MV4-11) bearing MLL fusion genes MLL-AF9 and MLL-AF4 respectively. Expression of EphA1, EphA2, EphA3, EphA7 and total Eph, as determined by ephrinA5-Fc, was analysed using flow cytometry. The results showed significant but variable expression of EphA1 and EphA3, and moderate expression of EphA2 and EphA7. Importantly, expression of total Ephs as determined by ephrinA5-Fc staining was significant on all samples (Figure 3.5). Together these data showed variable level of Eph expressions on patient samples and leukemic cell lines with MLL rearrangement. In particular, EphA2 was expressed at different levels in individual leukemic samples but the significant expression in a proportion of clinical

samples bearing the 11q23 chromosomal (MLL) re-arrangement, suggested that EphA2 might be a therapeutic target in human MLL-driven leukaemias.

A




B

| | Patient 1 B-ALL | Patient 2 B-ALL | Patient 3 T-ALL | Patient 4 AML | Patient 5 AML | Patient 6 AML | Patient 7 B-ALL | Patient 8 AML | THP-1 | MV4-11 |
|---|---|---|---|---|---|---|---|---|---|---|
| EphA1 | + | + | +++ | - | ++ | - | - | + | - | + |
| EphA2 | + | - | + | - | ++ | - | + | - | ++ | - |
| EphA3 | - | + | - | - | +++ | +++ | ++ | - | +++ | ++ |
| EphA7 | + | ++ | - | + | - | - | + | + | ++ | - |
| ephrinA5-FC | + | ++ | + | + | +++ | +++ | + | +++ | +++ | +++ |

**Figure 3.5. Expression analysis of EphA1, EphA2, EphA3 and EphA7 subfamily and ephrinA5-Fc on patient samples and human leukemic cell lines with MLL-rearrangement.** (A) Representative overlay of flow cytometric analysis of EphA1, EphA2, EphA3, EphA7 and ephrinA5-Fc on patient samples and human cell lines. (B) This table represents the expression level of Eph receptors on patient samples (patients 1-8) and human leukaemia cell lines (THP-1 and MV4-11).

### 3.3.4 MLL-AF9 leukemic mice treated with EphA2 monoclonal antibody or radiolabelled conjugated EphA2 antibody

Whilst EphA2 expression is not functionally important for the leukemic process, the expression has been detected on MLL-AF9 leukemic mice indicating that this protein might be a potential therapeutic agent in the treatment of MLL-AF9 leukaemias. To test this possibility I have used the EphA2 monoclonal antibody (IF7 mAb), which binds at nanomolar affinity to both mouse and human EphA2, in the treatment of high EphA2 expressing MLL-AF9 secondary transplanted leukemic mouse model. When

C57BL/6 mice were injected with different doses of IF7 every 48 hours, it had been shown previously that serum levels >1 mg/ml, a level predicted to give saturation binding of EphA2, were sustained for at least 48 hours when doses of 0.1 mg or greater were administered intraperitoneally. Thus, the MLL-AF9 mice were treated with 0.1 mg of IF7 mAb or vehicle only (PBS) control every second day until the mice were euthanized based on the severity of the disease. The results showed that there were no statistical difference in survival and engraftment of leukemic mice treated with IF7 mAb compared to vehicle only PBS control (Figure 3.6A, B). As the antibody had no significant effect, I next examined whether it could be used to deliver a radioactive "payload" to the leukemic cells. To verify this, the EphA2 mAb conjugated to radiolabelled Lutetium-177 (Lu-IF7) was utilised in the treatment of MLL-AF9 leukemic mice. The mice bearing $GFP^+$ MLL-AF9 leukaemia were treated with two separate doses of 3 MBq/10 mg of Lu-IF7 mAb, unlabelled IF7 mAb or PBS only control, 21 and 23 days after transplantation with MLL-AF9 leukemic cells from primary transplant. A significant reduction in blood engraftment was observed in Lu-IF7 treated mice compared to PBS treated control (Figure 3.6D). There were also significant improvement in the survival of Lu-IF7 treated mice compared to IF7 mAb and PBS treated control group (Figure 3.6E). More importantly, control mice with no leukemic cells exhibit no significant weight loss or clinical signs of toxicity when treated with Lu-IF7 mAb at the specific treatment dose. In a further experiment I also looked at the non-specific effect of Lutetium-177 on the MLL-AF9 leukaemia by treating the mice with radiolabelled EphA3 mAb (Lu-IIIA4), which shows no specific binding to the leukemic cells. Again, the Lu-IF7 mAb showed a prolonged survival but there were no significant differences between PBS, IF7 unlabelled and the Lu-IIIA4 treatment groups (Figure 3.6F). These data suggest that EphA2 may be a therapeutic target for some cases of MLL-driven leukaemia.

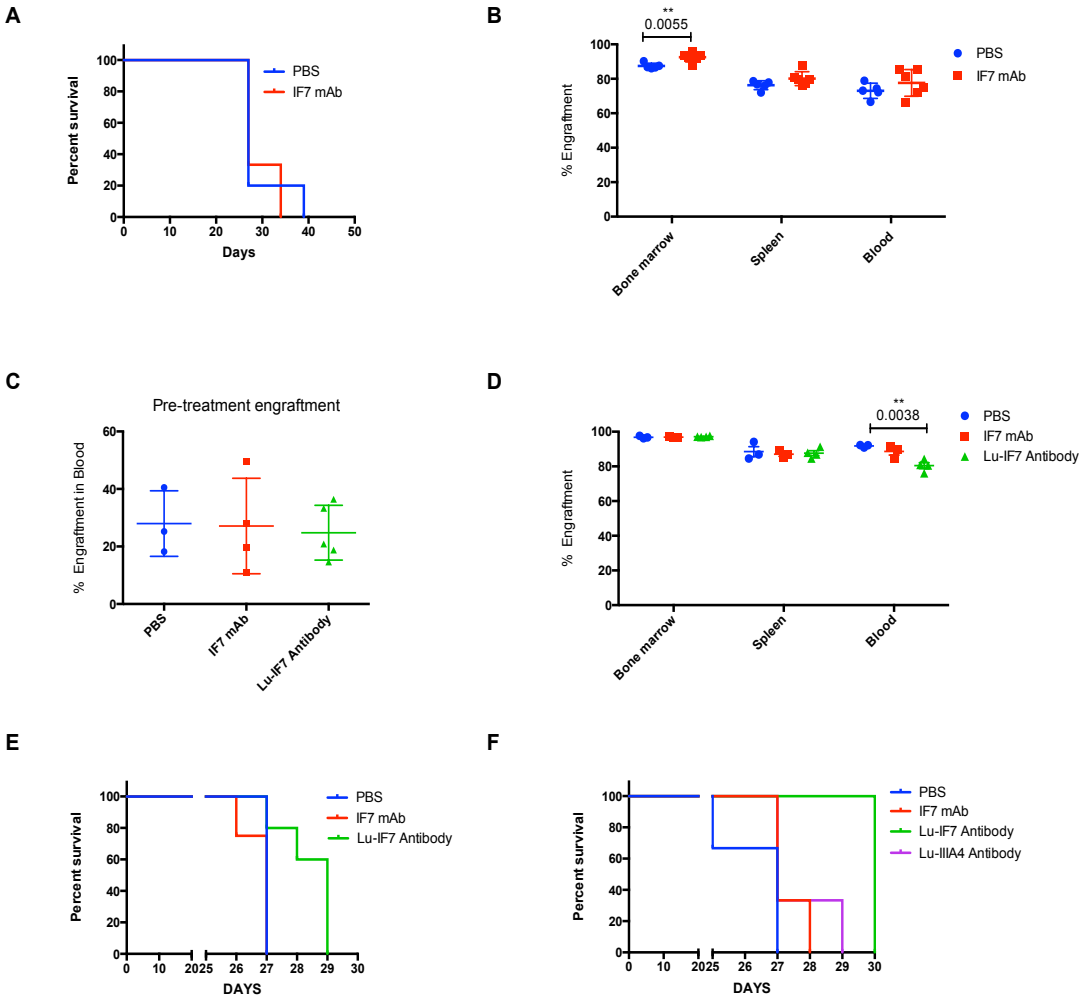



**Figure 3.6. Survival and engraftment data from MLL-AF9 leukemic mice treated with PBS, IF7 mAb, radiolabelled EphA2 (Lu-IF7) or radiolabelled EphA3 (Lu-IIIA4) antibody.** (A) Survival of MLL-AF9 leukemic mice treated with IF7 mAb or PBS control showed no significant differences between the two groups. (B) Percentage of $GFP^+$ cells in bone marrow, spleen and blood showed significantly higher $GFP^+$ cells in bone marrow of IF7 mAb treated mice compared to PBS control (P value. 0.0055). There were no significant differences observed in spleen and blood of the IF7 treated MLL-AF9 mice compared to wild type control mice at time of cull (n= 5 PBS treated, n=6 IF7 treated). (C) Percentage of $GFP^+$ cells in blood of MLL-AF9 leukemic mice 21 days post transplantation and prior to the antibody treatment. (D) Percentage of $GFP^+$ leukemic cells in bone marrow and spleen of the MLL-AF9 mice treated with PBS, IF7 mAb and Lu-IF7 mAb didn't show any significant differences in engraftment, however there were significantly less blood engraftment in the Lu-IF7 treated group compared to PBS treated mice (P value. 0.0038). (E) Survival of MLL-AF9 leukemic mice treated with PBS, IF7 mAb and Lu-IF7 antibody showed no significant differences between PBS and IF7 mAb treated group. Significant increase in the survival of Lu-IF7 antibody treated group was observed compared to PBS (P value. 0.0404) and IF7 mAb treated group (P value. 0.0201) (n= 3 PBS

treated, n=4 IF7 mAb treated and n=5 Lu-IF7 treated). (F) Survival of MLL-AF9 leukemic mice treated with PBS, IF7 mAb, Lu-IIIA4 or Lu-IF7 antibody showed no significant differences between PBS, IF7 and Lu-IIIA4 antibody treated groups. Significant increase in the survival of Lu-IF7 mAb treated group was observed compared to PBS (P value. 0.0140), IF7 mAb (P value. 0.0100) and Lu-IIIA4 mAb (P value. 0.0100) treated group (n= 3 PBS treated, n=3 IF7 mAb treated, n=3 Lu-3A4 antibody and n=4 Lu-IF7 treated). The data represent mean ± SEM. (n) represent the number mice used in each experiment. Unpaired *t* test was performed for statistical analyses.

## 3.4 Discussion

In this chapter I initially analysed expression of EphA1, EphA2 and EphA7 in the bone marrow cells from GFP-control mice, BCR-ABL and MLL-AF9 leukemic mice. My results clearly showed detectable levels of both mRNA and protein expression of EphA2 and EphA7 in the MLL-AF9 leukemic cells compared to negligible expression of EphA2 and EphA7 in BCR-ABL leukaemia or GFP-control. Thus, I subsequently focused on the MLL-AF9 model only. I investigated the possible functional effects of EphA2 expression on MLL-AF9 type leukaemia along with the capacity of EphA2 mAb as a targeted antibody therapy or radio-immunotherapy. As previously mentioned, EphA2 is expressed on variety of different cancers and studies by Nakanishi et al. [113] showed expression of EphA2 mRNA along with other members of Eph family on MLL-AF9 transfected K562 cells.

To test whether EphA2 was functionally important in the MLL-AF9 model, I looked at the effect of EphA2 deficiency on progression and severity of MLL-AF9 type leukaemias. When MLL-AF9 transduced haematopoietic stem cells derived from either wild type or EphA2 knockout mice were injected into wild type recipients they gave rise to leukaemias with comparable disease latency and severity. To explore any effect of EphA2 knockout on LSC self renewal, secondary transplantation experiments were performed in which the secondary recipient mice were transplanted with EphA2 knockout or wild type MLL-AF9 leukemic cells, and the results again showed no effect on survival, implying no functional effect of EphA2 in LSCs. Using specific markers, I have also checked cell cycle and apoptosis of the EphA2 knockout leukemic cells compared to wild type control. My results showed no significant differences in cell cycle between the two groups. However, the apoptotic assay showed an increase in the number of necrotic cells in the EphA2 knockout MLL-AF9 spleen compared to wild type control. EphA2 expression has been associated with

angiogenesis in solid tumours [99] hence, whilst not affecting disease outcome, the increased number of necrotic cell could be the result of reduction in angiogenesis in the absence of EphA2 expression. Overall, my results showed that EphA2 is not essential for MLL-AF9 induced leukaemogenesis.

Whilst these findings suggest that EphA2 itself has no direct functional role in MLL-AF9 leukaemia, this does not exclude an Eph-mediated effect, as there may be compensation from other Eph proteins. Based on the published literature, the most obvious candidate was EphA7 and as shown in Figure 3.4, expression of EphA7 was detected on both EphA2 knockout and wild type MLL-AF9 cells, although the expression level was not higher in the EphA2 knockout leukemic cells. On the other hand, ephrinA5-Fc staining was utilised as an indicator of total EphA expression in the MLL-AF9 leukaemia, it was notable that wild type control and EphA2-null leukemic cells had similar levels of ephrinA5-Fc expression. This suggests that in the absence of EphA2, there is a compensatory increase in expression of other members of EphA family such that the total capacity for Eph signalling is unchanged. Thus, a role for Eph receptors in MLL-AF9 leukaemia cannot be excluded. Therefore it would be of interest to examine this leukemic model in the mice with more than one Eph knockout, and in particular EphA2/EphA7 double knockout mice. However, at this stage these mice are not available for analysis and thus this is beyond the scope of this thesis.

As a prelude to exploring the possibility of targeting EphA2 in MLL-driven leukaemia, I performed a limited analysis of AML and ALL leukaemias from human patient samples and human leukemic cell lines with MLL rearrangement. As in the mouse leukaemias, EphA2 expression was variable but some cases showed moderate levels of EphA2 expression along with expression of other EphA proteins including EphA1, EphA7 and EphA3. This analysis suggested that pre-clinical testing of anti-EphA2 mAb therapy in the MLL-AF9 murine leukaemia model would potentially inform the possibility of clinical translation. EphA2 mAb treatment has been previously used in different types of cancers that express EphA2 and has recently been reviewed by Boyd et al. [157]. Therefore, I hypothesised that treatment with EphA2 mAb maybe effective in reducing the latency and the severity of MLL-AF9 type leukaemia. My findings indicate that treatment with unmodified IF7 mAb had no

effect on tumourigenicity and latency of the MLL-AF9 leukaemia. This may not be surprising as IF7 is an IgG1 mAb and it is not expected to mount a significant Fc-mediated cytotoxic response, therefore any effect would have to be a direct effect on EphA2 function. Given the expression of other Eph receptors to compensate for any effect on EphA2, as discussed in the context of the EphA2 knockout, even an inhibitory effect of IF7 on EphA2 might have no overall effect on the leukemic cells.

Recently the use of monoclonal antibodies conjugated to target a radionuclide to the cancer cells but not other normal cells have been studied extensively. Previous studies in non-Hodgkin's lymphoma using monoclonal antibodies conjugated to $^{131}$I-tositumomab and $^{90}$Y-ibritumomab tiuxetan have shown promising effects as treatment option in this type of leukaemia [187]. Therefore I examined the EphA2 antibody as a target for delivering cytotoxic "payload" to the EphA2 expressing leukemic cells. To do this, I used the EphA2 mAb radiolabelled with lutetium-177 to directly target leukemic cells in MLL-AF9 mice. Reassuringly, there were no significant toxicity observed in these mice, despite concerns raised by trial of an EphA2 antibody conjugated to a drug which was halted due to bleeding complications [188]. My results showed a significant increase in the survival of the $^{177}$Lu-IF7 treated mice along with significant reduction in blood engraftment. In this study the increase in survival, whilst relatively small, is significant in this highly malignant, treatment-refractory, leukaemia model. The magnitude of the effect may also be compromised by the fact that these mice were treated in the late stages of the disease and with only two doses. Therefore, in future studies I will be required to treat these mice when blasts first become detectable in the blood and also to study multiple dosing regimens and combination with anti-leukemic drugs. Notably, this result is consistent with recent studies by Day et al. (2013) where effective targeting of tumourigenic cells was achieved upon treatment with lutetium-177 conjugated-EphA3 mAb in glioblastoma [59].

In conclusion, I found that the presence of EphA2 expression is not essential for leukaemogenesis, perhaps due to functional redundancy between members of Eph family of receptor tyrosine kinases. However, targeting MLL-AF9 leukaemia with radiolabelled EphA2 could be a successful approach in treatment of EphA2-positive leukaemias as EphA2 is not strongly expressed on normal haematopoietic cells. I also

showed that by using $^{177}$Lu-IF7 anti-EphA2 mAb it was possible to specifically target the leukemic cells with minimal toxicity.

# Chapter 4 . EphA3 AS A TARGET FOR MONOCLONAL ANTIBODY TREATMENT IN ACUTE LEUKAEMIA

## 4.1 Introduction

Eph receptor tyrosine kinase proteins interact with cell-surface ephrin ligands to regulate cell growth, positioning and migration by modulating cell shape and adhesion. Both Eph and ephrin proteins are important in regulating cell-cell interactions and their engagement can initiate either cell adhesion or repulsion in different cellular contexts. Cell repulsion occurs when bidirectional signalling triggers cytoskeletal collapse and loss of focal adhesions, resulting in cell rounding and cell detachment while cell attachment occurs when signals are in favour of cell adhesion and migration [68].

Eph and ephrin genes are strongly expressed on various embryonic tissues and they are involved in many developmental processes, particularly in formation of tissue boundaries, development and assembly of neuronal circuits and also in the development of vasculature [71]. Eph/ephrin genes also have an important role in adult tissues and mainly in organ pathogenesis, regeneration and maintenance [157]. High expression of Eph genes have been observed in many cancers and over-expression could have both tumour promoting and inhibiting effect depending on the tumour type, type of Eph receptor, kinase activity and the stage of disease [157]. For instance, the expression of Eph and ephrin has been linked to invasiveness and metastatic spread of the tumour cells.

EphA3 was originally identified in LK63 pre-B acute lymphoblastic leukemic (ALL) cells, which show high expression, and has been shown to be highly expressed on embryonic tissues and it is involved in different stages of embryonic developments including lung, kidney, brain, spinal cord and central nervous system [103,144,189,190]. The most notable expression was observed in developing heart, a finding motivated by the observation that EphA3 knockout mice have cardiac defects and 75% of these mice die of cardiac failure [191]. EphA3 is expressed at very low levels in adult tissues but this expression is high in many different malignancies including sarcomas, lung cancer, melanoma, glioblastoma and leukaemia [157]. EphA3 expression has been identified in T-cell leukaemia cell lines including Jurkat, Molt-4, JM and HSB-2, while expression was absent in normal T-cells. Studies by Smith et al. (2004) suggested that EphA3 may have a role in malignant T-cells adhesion and motility, as they showed loss of cell adhesion to fibronectin upon ephrinA5 stimulation of Jurkat

cells and regulation of EphA3 expression via CD28 or insulin-like growth factor 1 receptor (IGF-1R) signalling [192]. As mentioned previously, significantly elevated EphA3 expression, compared with normal blood cells, was detected in a proportion of lymphoid and myeloid malignancies [103]. Studies by Guan et al. (2011) showed presence of copy-number variations (CNVs) of EphA3 in various haematological malignancies and recent studies on primary leukemic mouse models and human leukaemias showed EphA3 as a cooperative response gene (CRG) responsible for leukaemia stem cell growth and survival [148,149]. Previous studies have shown that EphA3 can induce both adhesive and cell repulsive responses in different cell types [68,145]. By analysing ephrin-induced cell adhesion in LK63 leukemic cells, a critical role was identified for protein phosphatase activity, which prevented EphA3 phosphorylation and hence maintained the Eph/ephrin adhesive bond and prevented initiation of the signalling mechanisms leading to cell repulsion [68].

The monoclonal antibody (IIIA4) used to isolate EphA3 protein was raised by immunising mice with LK63 pre-B ALL cells. This antibody was the key for identifying EphA3 (Hek) as a new member of the Eph family [103,144]. The IIIA4 antibody binds with high affinity to the EphA3 protein close to the ephrin-binding site [55,193]. Previous studies showed that treatment of EphA3 on HEK293 cells with pre-clustered IIIA4 could induce activation of EphA3 thereby causing cytoskeleton contraction and cell rounding [146]. EphA3-specific monoclonal antibody, IIIA4, binds and activates both human and mouse EphA3 with similar affinities and binding is followed by internalization of receptor-antibody complexes [146]. Through these studies EphA3 was identified as a potential therapy target for leukaemia and KaloBios Pharmaceuticals Inc. developed a Humaneered® derivative of the original IIIA4 anti-EphA3 monoclonal antibody. This antibody is known as KB004, and a clinical trial has commenced which targets EphA3 protein using this antibody in acute leukaemia.

Recent studies by Day et al. (2013) investigated the tumourogenic and therapeutic role of EphA3 in glioblastoma. In this study EphA3 knockdown glioblastoma (GBM) cell lines showed reduced tumourigenicity. Consistent with this finding it was shown that treatment with radiolabelled IIIA4 antibody induced tumour regression in xenograft model of GBM [59].

In this chapter I looked at the role of EphA3 expression in the development and spread of leukaemia in leukemic NOD/SCID mice. In particular, I analyse the effect of the IIIA4 monoclonal antibody treatment in EphA3 positive and negative leukemic mouse models to explore the functional effect of this antibody and to examine if this direct effect can be amplified by antibody targeting of a cytotoxic payload.

## 4.2 Methods

### 4.2.1 Cell culture

All the leukemic cell lines, including LK63, Reh, Jurkat, K562, Molt-4, NB-4, SKM-1, THP-1, U937 and MM6, were maintained in RPMI, 10% foetal calf serum (FCS) medium. Stable firefly luciferase-transfected Reh (Reh/Luc) and LK63 (LK63/Luc), Reh EphA3-expressing (Reh/A3) and LK63 EphA3-knockdown (LK63/A3KD) cells were maintained in RPMI, 10% FCS and 0.5 μg/mL puromycin medium.

### 4.2.2 Antibodies and Eph fusion proteins

The following monoclonal antibodies generated in our laboratory were used in this study: 3E9 anti-EphA1, 1F7 anti-EphA2, IIIA4 anti-EphA3 and 1F9 anti-EphA4, all are IgG1k, antibodies. Anti-EphB4 monoclonal antibody clone 395810 was purchased from R&D Systems (FAB3038P). For therapeutic targeting of EphA3, a humanised chimeric version of IIIA4, in which the $V_H$ domain of the IIIA4 was inserted into a human IgG1 antibody backbone (provided by Prof. Andrew Scott, Ludwig Institute) was employed.

EphA3-Fc and EphB4-Fc, fusion proteins, were used in which the coding regions for the Eph or ephrin extracellular domains were fused to the hinge and Fc regions of the heavy chain of human IgG1 as previously described [194]. The resulting expression vectors were transfected into Chinese Hamster Ovary (CHO) cells to obtain stably expressing cell lines. Fusion proteins were purified from supernatants of CHO cell transfected lines by protein-A sepharose chromatography [195]. EphA3-Fc and EphB4-Fc were utilised for analysis of total ephrinA and ephrinB expression in leukemic cells.

### 4.2.3 Flow Cytometry

To analyse cell surface expression of Eph proteins, $2\times10^6$ cells were washed in 2% foetal bovine serum/phosphate buffered saline (FBS/PBS). The cells were stained with 10 μg of anti-EphA1, -EphA2, -EphA3 and -EphA4 monoclonal antibodies directly conjugated to Alexa Fluor 647 or Phycoerythrin (PE)-conjugated anti-EphB4 monoclonal antibody (R&D Systems, FAB3038P), and incubated on ice for 30 minutes in the dark. Cells were washed twice with 2% FBS/PBS and stained with SYTOX-Blue (Life Technologies) dead cell marker and analysed on LSRFortessa (BD Biosciences).

For analysis of the ephrin ligand expression aliquots of $2\times10^6$ LK63 or Reh cells were stained with EphA3-Fc or EphB4-Fc fusion proteins. The cells were washed twice with 2% FBS/PBS and stained with Alexa fluor 488 goat anti-human IgG (A-11013, Life Technologies) as a secondary antibody. The cells were washed twice with 2% FBS/PBS and analysed on LSRFortessa (BD Biosciences).

To analyse the leukemic cell engraftment, blood, bone marrow and spleen tissues were collected from mice that were euthanized according to procedures approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee. Blood samples were obtained using submandibular blood sampling technique and lysed with red blood cell lysis buffer (Pharm Lyse™, BD Biosciences). In order to obtain bone marrow cells, both femurs from mice were flushed with a 26G needle and syringe containing 2% FBS/PBS and passed through 100 μm mesh filters. Spleens were dissociated into single cell suspensions and passed through a 70 μm mesh filters and red blood cells were lysed using red cell lysis buffer (Pharm Lyse™, BD Biosciences). Blood, bone marrow and spleen cells were counted and stained with R-phycoerythrin/Cyanine dye (PE/Cy7) anti-human CD45 (Biolegend) and Allophycocyanin (APC) anti-mouse CD45 (Biolegend) antibodies to enable separate enumeration of human and mouse haematopoietic cells. Then SYTOX-Blue (Life Technologies) was utilised to exclude dead cells. Data acquisition was performed using LSRFortessa flow cytometer (BD Bioscience) and the data were analysed using FlowJo software (Tree Star).

### 4.2.4 Reverse transcriptase polymerase chain reaction

RNA was isolated from leukemic cell lines using the RNeasy Mini Kit (QIAGEN) and complementary DNA (cDNA) was synthesized using SuperScript III (Life Technologies) with respect to the manufacturer's instructions. Relative transcript quantification was performed with SYBR Green QPCR Mastermix (Applied Biosystems) using the RotorGene 3000 (QIAGEN). Real-Time PCR System and gene expression was calculated relative to β-actin as the housekeeping reference gene.

### 4.2.5 The leukemic xenograft model

The LK63, Reh, LK63/A3KD, Reh/A3, LK63/Luc and Reh/Luc cell lines were used to establish leukemic engraftment xenograft models in the NOD/SCID mice. The mice were injected intravenously into the tail vein with $5\times10^6$ cells for the leukemic engraftment model. A leukemic intrafemoral mouse model was established in which NOD/SCID mice were injected in the right femur with $2\times10^5$ leukemic cells. These xenograft models were treated intraperitoneally with humaneered anti-EphA3 monoclonal antibody IIIA4 (IIIA4 mAb), control IgG antibody or vehicle (PBS), starting on the day of the cell transfer and then repeated on alternate dates until the mice were euthanized due to progression of the disease. These mice were monitored for disease progression and signs of illness and scored accordingly. All mice were scored individually based on weight loss (Score 0-2), body posture (Score 0-2), activity level (score 0-2), and grooming (score 0-2) and were subsequently euthanized once the total score was 7 or greater.

### 4.2.6 In vivo bioluminescence imaging

Anaesthetised mice were injected with 500 µg of firefly luciferin (PerkinElmer) subcutaneously. Mice were imaged 5 minutes later using the Xenogen imaging system IVIS 100 (PerkinElmer).

### 4.2.7 Activation assay, western blot and microscopy

Western blot analysis was used to evaluate phosphorylation states of Src (phospho-Src-419, phospho-Src-527, 1:1000, Cell Signaling), Akt (phospho-Akt, 1:1000, Cell Signaling), Erk (phospho-Erk 1:1000, Cell Signaling) and total Src (1:1000, Cell Signaling) in LK63 and LK63/A3KD cells. Aliquots of 1.5 µg/ml of IIIA4, human IgG controls and ephrinA5-Fc were pre-clustered with 0.75 µg/ml of anti-human IgG

(huIgG) at 4°C for 1 hour. $1\times10^6$ LK63 and LK63/A3KD cells were incubated in serum free media for minimum of 4 hours. These cells were then incubated in PBS or PBS containing 1.5 μg/ml of IIIA4, pre-clustered human IgG controls, pre-clustered IIIA4 or pre-clustered ephrinA5-Fc and incubated at 37°C for 20 minutes after which they were pelleted and resuspended in 100 μl of ice cold lysis buffer (150 mM NaCl, 1% Triton X-100, 5mM EDTA pH8, 10mM Tris-Cl pH7.4, 0.1 mM PMSF and cOmplete Protease Inhibitor Cocktail Tablets (Roche)). The lysates were centrifuged at 15,000 rpm to remove cell nuclei and debris and the protein concentration of the cleared lysate was determined by Bradford (Biorad) protein assay. Protein lysate at final concentration of (80 μg/sample) was mixed with appropriate volume of 5x sample buffer (130 mM Tris-Cl pH8.0, 20% (v/v) glycerol, 4.6% (w/v) SDS, 0.02% bromophenol blue, 2% DTT and 5% β-mercaptoethanol) and boiled for 5 minutes. Samples were run on denaturing SDS-PAGE gels (10% acrylamide) at 120 V for 1 hour and transferred overnight at 30 V onto Immuno-Blot™ PVDF Membrane (BioRad). The membrane was blocked with 5% skim milk powder in TBST (137 mM NaCl, 10 mM Tris, pH7.4, 0.02% Tween 20) for 1 hour and incubated with primary antibody diluted in 5% skim milk powder in TBST for a minimum of 1 hour. The membrane was then washed three times with TBST for a total of at least 30 minutes before incubation for 1 hour in secondary detection antibody. Either anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (HRP; 1:4000, Amersham Biosciences) was used as a secondary detection antibody. The membrane was then washed for 1 hour and the protein was detected using ECL™ Western Blotting Detection Reagents as per manufacturer's instruction (Amersham Biosciences).

For live cell imaging, $1\times10^6$ LK63 and LK63/A3KD cells were seeded onto fibronectin-coated plates for 24 hours prior to imaging. The cells were incubated in $CO_2$-independent serum-free medium (Life Technologies) for a minimum of 4 hours and activated with IIIA4, pre-clustered human IgG control, pre-clustered IIIA4 or pre-clustered ephrinA5-Fc. The cell were imaged every 10 seconds for 20 minutes using an Olympus IX81 Microscope (Olympus).

### 4.2.8 Radiolabelling and immunoreactivity

Preparation and testing of the radiolabelled IIIA4 antibody was carried out in Prof. Andrew Scott's laboratory at the Ludwig Institute, Melbourne.

Bismuth-213 ($^{213}$Bi) was eluted from an Actinium-225 generator obtained from the Institute of Power Engineering and Physics, Obninsk, Russia. Radiolabelling of IIIA4 and isotype control antibody with $^{213}$Bi was achieved using the bi-functional metal ion chelate C-functionalised *trans*-cyclohexyl-diethylenetriaminepentaacetic acid (CHX-A″-DTPA) [196]. The immunoreactivity of radiolablelled IIIA4 was determined by single point binding assays. The LK63 cells ($10\times10^6$) were incubated with 20 ng radiolabelled $^{213}$Bi-IIIA4 for 30 minutes at room temperature. Cells were washed three times, and immunoreactivity was expressed as a percentage of activity in duplicate radioconjugate standards.

### 4.2.9 $^{213}$Bi-IIIA4 biodistribution study

To assess the biodistribution of the radioconjugates in leukaemia, mice were injected with $^{213}$Bi-IIIA4 or $^{213}$Bi-isotype control in 0.1 ml saline 14 days after injection of LK63 cells. To assess the biodistribution of $^{213}$Bi-IIIA4, groups of 4 mice were culled at 5 minutes, 1 hour, 2 hours and 3 hours post radioconjugate injection. At each time point, the mice were euthanized according to institute guideline by isofluorane anaesthesia and then immediately bled via cardiac puncture. Blood and tissues (liver, spleen, kidney, muscle, skin, bone, lung, heart, stomach, brain, small bowel and colon) were resected, blotted dry for weighing and radioactive counting. For controls, $^{213}$Bi-labeled isotype control was similarly injected into leukaemia-engrafted mice (n=4) and sacrificed 1 hour after injection for blood and tissue collection. The radioactivity of all samples was measured in a dual gamma scintillation counter (Cobra II Auto Gamma; Packard Instruments). The results of radiolabelled antibody biodistribution over time were calculated as the percentage injected dose per gram of blood or tissue (%ID/g) and were expressed as the mean ± standard deviation for each time point.

### 4.2.10 $^{213}$Bi-IIIA4 therapy study

To assess the efficacy of $^{213}$Bi-IIIA4 $\alpha$-emitter radioimmunotherapy in leukaemia, two therapeutic regimens were explored using mice injected with $13\times10^6$ LK63 cells.

In the first study 6 days after injection of LK63 cells, mice were injected by tail vein injection with single doses of $^{213}$Bi-IIIA4 or controls. In the second study mice received multiple doses of $^{213}$Bi-IIIA4 or controls and the treatment injection were performed on day 2, 3, 5 and 6 after LK63 cells injection. In both studies treatment groups comprised individual $^{213}$Bi-IIIA4 doses of 12.5 μCi (115 mg protein) or 25 μCi (180 μg protein) or $^{213}$Bi- isotype control at doses of 12.5 μCi (75 mg protein) or 25 μCi (150 μg protein), delivered in 0.1-0.3 ml saline. Accordingly the multi-dose treatment groups received a total of 50 or 100 μCi radiolabel test or control antibody. Control groups received saline vehicle or 180 μg unlabelled IIIA4 per dose (total, 720 mg protein) to match protein dose of monoclonal antibodies across all therapy groups, in equivalent volumes to the radiolabelled antibodies.

### 4.2.11 Statistics

Statistical analyses were performed using GraphPad Prism Version 6.01 software. Data are shown as mean ± SEM of at least three replicates, unless stated otherwise. Statistical significance was determined using an unpaired *t* test. The survival of the control and the treatment groups were compared using Kaplan–Meier analysis and statistical differences analysed using the log rank test.

## 4.3 Results

### 4.3.1 Expression of Eph receptors and their ephrin ligands in leukaemia

Monoclonal antibodies generated in our laboratory were used to examine expression of EphA1, EphA2, EphA3 and EphA4 in 22 clinical samples of acute leukaemia and two refractory anaemia samples with excess blasts (RAEB) using flow cytometry. These results confirmed that majority of both myeloid and lymphoid leukaemias expressed significant levels of EphA3, but little or no expression of either EphA1 or EphA4 was detected even in EphA3-negative cases; however, a small number of samples did express EphA2, but at lower levels compared to EphA3 (Table 4.1).

**Table 4.1. Analysis of EphA expression in patient samples.** (- indicates no expression , ± indicates low level of expression, + refer to moderate level of expression and ++ indicates strong expression.)

| Tumour type | Tissue | EphA1 | EphA2 | EphA3 | EphA4 |
|---|---|---|---|---|---|
| AML M0 (R24) | BM | - | - | ± | - |
| AML M0 (SM) | BM | - | - | ± | - |
| AML M1 (P4) | Blood | ± | + | ++ | ± |
| AML M1 (R22) | Blood | - | + | ++ | ± |
| AML M1 (R32) | BM | - | - | ± | - |
| AML M1 (CC) | BM | - | ± | ++ | - |
| AML M2 (CH) | BM | - | ± | - | - |
| AML M2 (R7) | Blood | - | ± | + | - |
| AML M2 (R26) | BM | - | - | ± | ± |
| AML M2 (P23) | BM | ± | + | ++ | + |
| AML M2 (P10) | BM | - | - | ± | - |
| AML M2 (P22) | BM | - | ± | ++ | + |
| AML M4 Eo (P16) | Blood | - | - | ++ | ± |
| AML M4 (R19) | BM | - | + | ++ | ± |
| AML M6 (R12) | Blood | - | - | ± | - |
| AML M7 (P18) | Blood | - | - | ± | - |
| AML multi dys (P21) | BM | +(s) | +(s) | ++ | +(s) |
| AML multi dys (R14) | BM | - | ± | ++ | - |
| RAEB (P15) | BM | - | - | - | - |
| RAEB (R15) | BM | - | ± | + | ± |
| Pre B ALL (P20) | BM | - | - | + | - |
| Pre B ALL (R31) | BM | ± | + | ++ | - |
| Pre B ALL (R34) | Apheresis | - | ± | ± | - |
| Pre B ALL (KY) | Apheresis | - | - | ± | - |

The expression of Eph and ephrin messenger RNA (mRNA) and protein levels were also assessed in a series of haematopoietic cell lines using RT-PCR and flow cytometric analysis. Similar to the results obtained from the clinical samples, EphA3 mRNA was the most prominent transcript detected. In the analysis of the leukemic cell lines EphA3 was again the most prominent transcript detected and levels of mRNA for all other EphA genes were negligible with the exception of EphA1 where low levels of expression were detected in some cell lines. Of all the EphB genes tested, EphB4 mRNA was detected in several lines, whereas EphB6 mRNA was only detected in T-cell lines, as previously reported (Figure 4.1A)[118,119,130]. Expression of

ephrin transcripts was generally low except for ephrinB2 and ephrinB1, which showed significant expression in Reh cells (Figure 4.1B).

EphA3 and EphB4 were two of the most prominent Ephs expressed on leukemic cell lines. This was confirmed by analysis of cell surface expression of EphA3 and EphB4 protein labelled with anti-EphA3 monoclonal antibody (IIIA4) or EphB4 monoclonal antibody and was performed using flow cytometric analysis. High expression of EphA3, which correlated with mRNA expression, was observed in LK63 and Jurkat, moderate EphA3 expression on Molt-4 and no expression on Reh cells, in keeping with previous data [101] (Figure 4.1C). EphB4 expression was assessed using anti-EphB4 monoclonal antibody, which showed high expression of EphB4 in Reh, LK63, U937 and K562 (Figure 4.1D). EphA3-Fc and EphB4-Fc fusion proteins were used for analysis of the ephrinA and ephrinB expressionon on the LK63 and Reh cells. Reh cells stained weakly positive for both EphA3-Fc and EphB4-Fc, which correlates with the expression of ephrinA and ephrinB mRNA in these cells (Figure 4.1E).

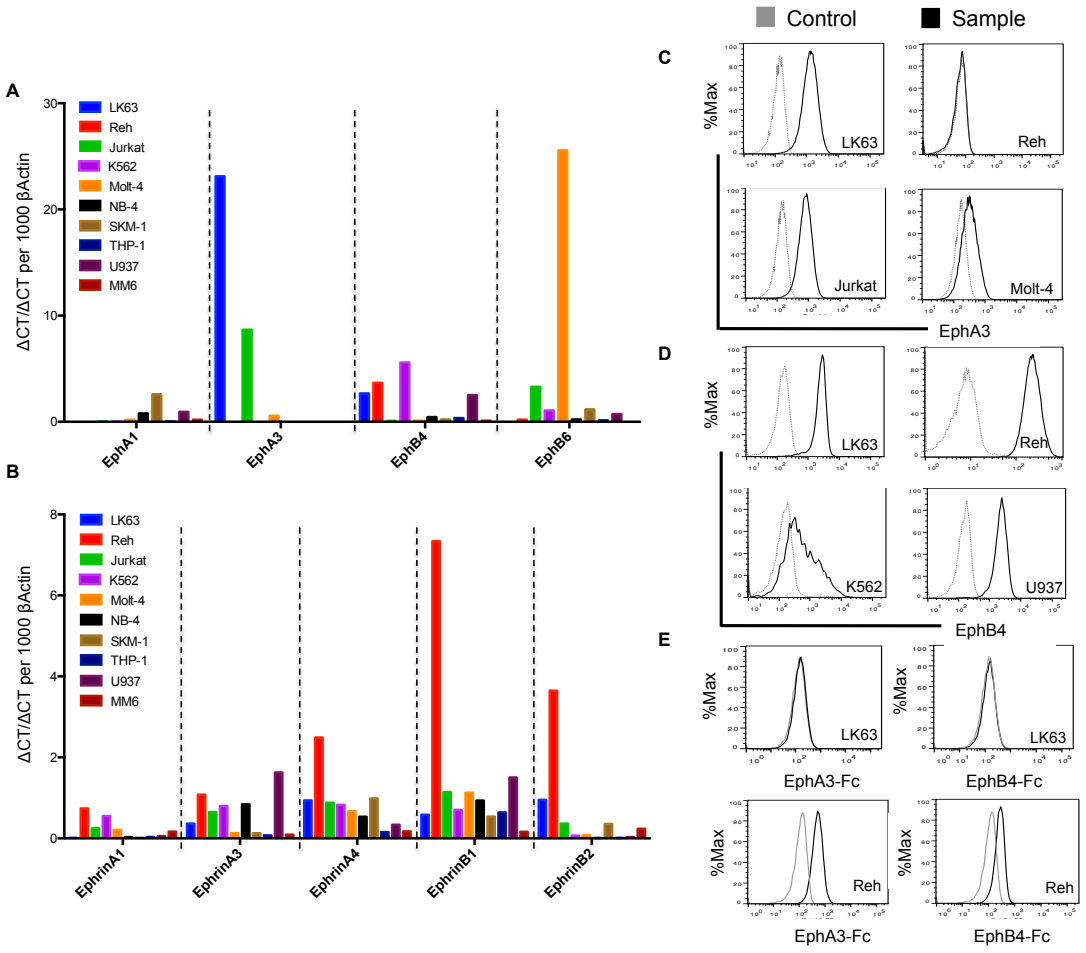


**Figure 4.1. Analysis of Eph and ephrin expression on leukemic cell lines.** (A) Expression of EphA1, EphA3, EphB4 and EphB6 in LK63, Reh, Jurkat, K562, Molt-4, NB-4, SKM-1, THP-1, U937, MM6 analysed per 1000 β-actin using RT-PCR. (B) Expression of ephrinA1, ephrinA2, ephrinA4, ephrinB2 and ephrinB3 in LK63, Reh, Jurkat, K562, Molt-4, NB-4, SKM-1, THP-1, U937 and MM6 analysed per 1000 β-actin using RT-PCR. (C) Cell surface expression of EphA3 in LK63, Reh, Jurkat and Molt-4 cells analysed using the anti-EphA3 monoclonal antibody (IIIA4). LK63 showed highest expression of EphA3 followed by Jurkat, Molt-4 and Reh with no EphA3 expression. (D) Cell surface expression of EphB4 in LK63, Reh, K562 and U937 cells analysed using the anti-EphB4 monoclonal antibody. High expression of EphB4 was observed in all of the leukemic cells tested. (E) Cell surface expression of ephrinA and ephrinB on LK63 and Reh cells using EphA3-Fc and EphB4-Fc fusion proteins.

### 4.3.2 Establishing Leukemic mouse models to investigate the role of EphA3 in leukaemia

To investigate the role of EphA3 in leukaemia, two pre-B ALL cell lines with similar characteristics and phenotype that differed in their EphA3 expression were chosen. Based on the expression analysis, LK63 cells showed the highest expression of EphA3, and Reh, did not express detectable level of EphA3, however both cell lines expressed comparable levels of EphB4. The LK63 cell line was derived from a child with pre-B ALL and its characteristics and phenotype ($CD10^{+}CD19^{+}CD20^{\pm}EphA3^{+}$) was described in detail previously [103]. The Reh cell line is also from a childhood pre-B ALL with similar characteristics and phenotype ($CD10^{+}CD19^{+}CD20^{-}EphA3^{-}$).

When injected into mice both cell lines reliably gave rise to leukemic engraftment and were thus selected as xenograft models to analyse the function and potential targeting of EphA3 in leukaemia.

The LK63 engraftment in bone marrow, spleen and blood was enumerated by flow cytometric analysis at different time points using human and mouse CD45 antigen to distinguish the relative number of LK63 cells and healthy mouse lymphocytes (Figure 4.2A). It was evident that LK63 cells preferentially grew in the bone marrow before significant numbers were observed in the spleen and peripheral blood (Figure 4.2B). The LK63 xenograft had significantly higher spleen weight compare to normal spleen (Figure 4.2C). Histological analysis showed that growth in the bone marrow was relatively uniform whereas the spleen initially showed foci of proliferating LK63 cells, which eventually became confluent and affected the normal splenic architecture (Figure 4.2D, E), consistent with the notion that the leukemic cells were seeded into

the spleen from the bone marrow. The Reh engraftment in bone marrow, spleen and blood was also analysed at different time points using flow cytometric analysis for human and mouse CD45 antigen. It was evident that Reh cells preferentially grew in the bone marrow followed by spread to the peripheral blood. Unlike LK63 xenograft, Reh xenografts showed minimal engraftment in the spleen with no significant differences in spleen weight at any time point after leukemic engraftment. Reh engraftment followed a somewhat slower time-course compared to LK63 xenografts (Figure 4.2F, G).

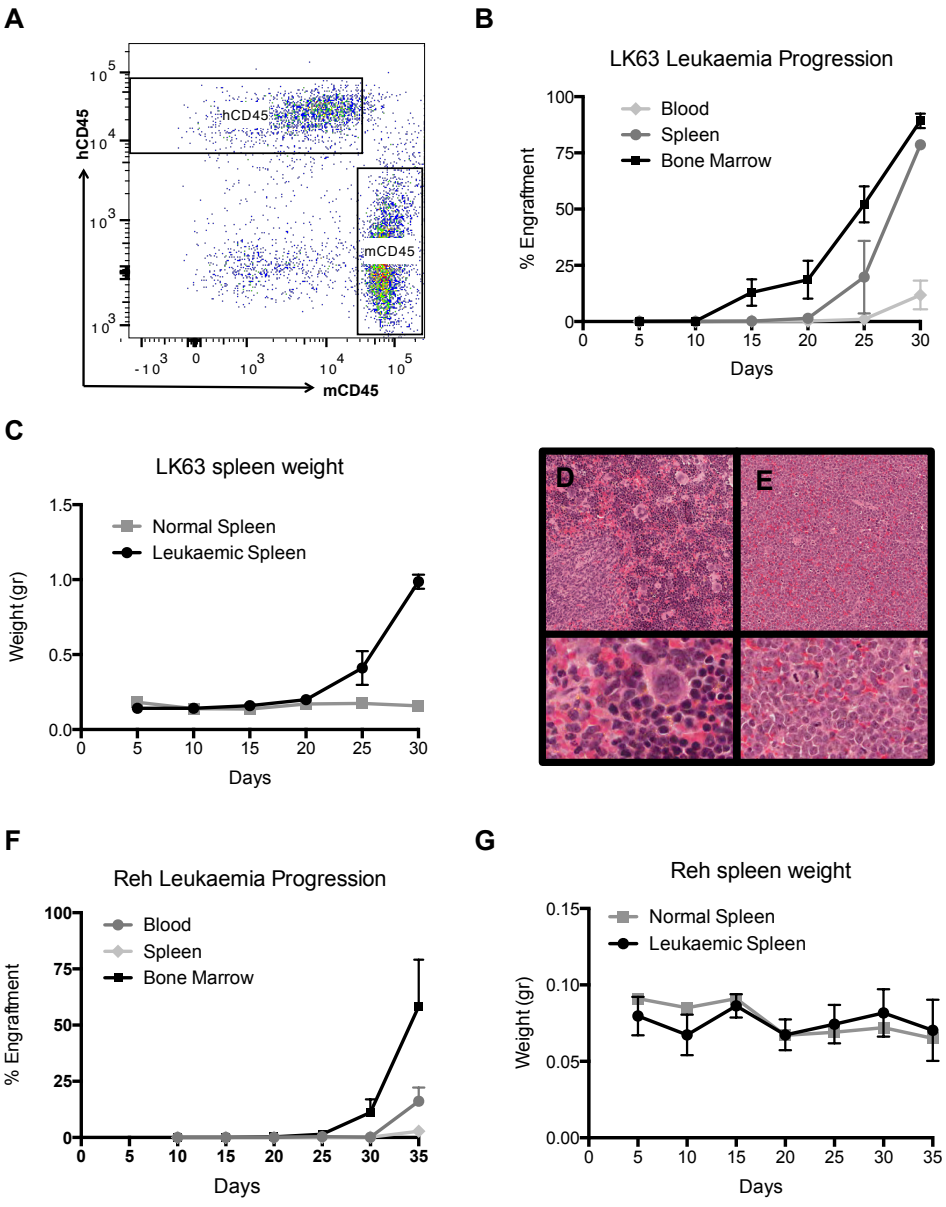


**Figure 4.2. Leukemic progression in LK63 and Reh xenograft model.** (A) Flow cytometry gating strategy for mCD45 and hCD45 in bone marrow, spleen and blood of leukemic mice. (B) Leukaemia progression in blood, spleen and bone marrow of LK63 leukemic mice was analysed by flow cytometry using hCD45 and mCD45 markers every 5 days for 30 days. (C) Spleen weight of LK63 leukemic mice compared to normal mice over 30 days in which the size of spleen became significantly enlarged in LK63 mice on day 25 and 30 post engraftment compared to normal control mice (n=3 LK63 mice, n=1 normal mice per time point). (D) Histology of normal spleen. (E) Histology of leukemic spleen from LK63 xenograft. (F) Leukaemia progression in blood, spleen and bone marrow of Reh-engrafted leukemic mice was analysed by flow cytometry using hCD45 and mCD45 markers for 35 days. (G) Spleen weight of Reh-engrafted leukemic mice compared to normal mice showed no changes in the spleen size over 35 days (n=3 LK63 mice, n=1 normal mice per time point).

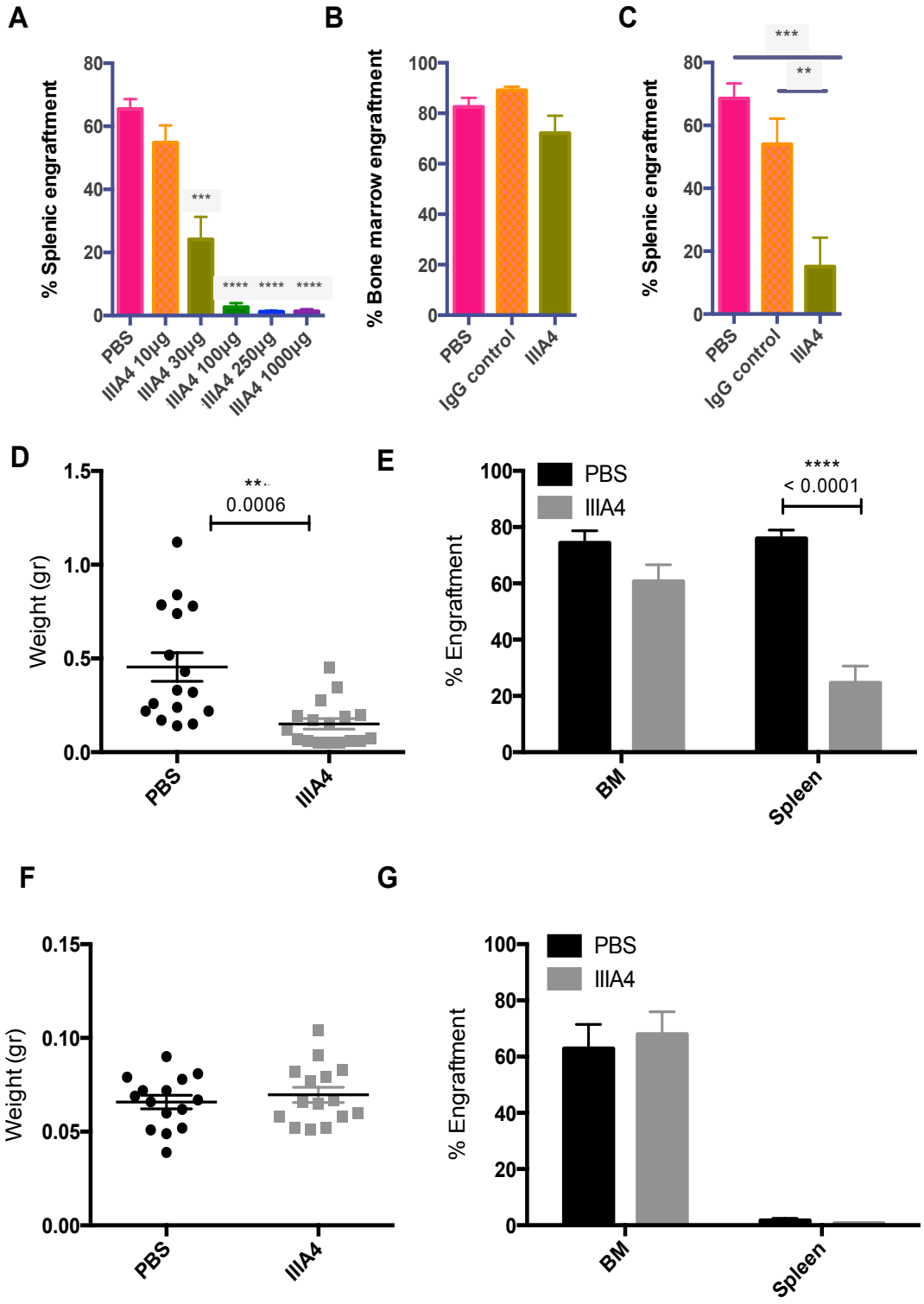
### 4.3.3 Treatment of leukemic mice with IIIA4 monoclonal antibody

In this section I describe experiments in which the LK63 and Reh xenografted mice were treated with the humaneered anti-EphA3 monoclonal antibody IIIA4 (IIIA4 mAb). Previous studies on IIIA4 tissue distribution showed effective targeting of EphA3-positive tumour cells with IIIA4 and negligible amounts of IIIA4 was observed in other tissues [146]. A dose response experiment was performed on LK63 xenograft model and the results showed optimum and maximum response in terms of reduction in spleen engraftment at a dose of 100 μg. Lower doses including, 10 μg and 30 μg were less effective compared to 100 μg. Higher concentrations at 250 μg and 1000 μg had similar therapeutic effect compared to 100 μg of IIIA4 treatment (Figure 4.3A). Therefore, in all of the subsequent experiments 100 μg of IIIA4 antibody was administered on alternate days starting immediately after LK63 leukemic cells injection.

Both LK63 and Reh xenografted mice were treated with 100 μg of IIIA4 mAb, vehicle only or control human IgG1 antibody (IgG control). In contrast to the IIIA4 treated group, no effect on engraftment were observed in either of the control groups. As there were no significant differences between PBS and human IgG treated controls, PBS was used as the negative control in subsequent experiments (Figure 4.3B, C).

The LK63 mouse model of leukaemia showed significant leukemic engraftment in bone marrow and spleen of the mice. Treatment of LK63 xenografted mice with IIIA4, significantly reduced engraftment in the spleen, as it was evident from lower

numbers of human CD45-positive cells in the spleen and reduced spleen weight. Engraftment of the LK63 cells in the bone marrow was also reduced compared to the PBS-treated control group (Figure 4.3D, E). A lesser increase in spleen weight, and reduced engraftment of spleen and bone marrow are indicative of reduced tumour growth in the LK63 xenograft upon treatment with IIIA4. In contrast, the Reh xenografted mice showed no therapeutic effect in response to treatment with IIIA4 compared to PBS treated control group, which is consistent with the absence of EphA3 on Reh cells. As mentioned previously, the Reh leukemic model, unlike LK63 xenografted mice, had minimal splenic engraftment. Treatment of Reh xenograft with IIIA4 showed no significant changes in splenic engraftment and in fact showed increased bone marrow engraftment compared to the PBS treated control group (Figure 4.3F, G).


A
% Splenic engraftment
80
60
40
20
0
***
**** **** ****
PBS
IIIA4 10µg
IIIA4 30µg
IIIA4 100µg
IIIA4 250µg
IIIA4 1000µg
B
% Bone marrow engraftment
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80
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0
PBS
IgG control
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C
% Splenic engraftment
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80
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Weight (gr)
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0.05
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G
% Engraftment
100
80
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40
20
0
PBS
IIIA4
BM
Spleen

**Figure 4.3. LK63 and Reh leukemic mouse models treated with PBS or IIIA4 mAb**. (A) Spleen engraftment in LK63 xenograft in response to PBS or IIIA4 treatment at 10 µg, 30 µg, 100 µg, 250 µg, and 1000 µg dosing. The engraftment was significantly reduced at 30, 100, 250 and 1000 µg of antibody treatment compared to the PBS-treated control group (***P value. 0.0007, ****P value. < 0.0001). No significant difference in engraftment was observed between 100, 250 and 1000 µg of IIIA4 dosing (n=4-9). (B) Percentage of bone marrow engraftment in the LK63 xenograft in response to PBS, IgG control and IIIA4 showed no significant differences between the three groups (n=8). (C) Percentage of spleen engraftment of the LK63 xenograft in response to PBS, IgG control and IIIA4 showed no significant differences between PBS and IgG control groups. There was a significant reduction in the spleen engraftment of IIIA4 treated group compared to PBS (P value. 0.0001) and IgG control (P value. 0.0069) treated groups (n=8). (D) Spleen weight for IIIA4 and PBS treated LK63 xenograft showed a significant reduction in spleen weight of IIIA4-treated mice compared to the PBS-treated control group (P value. 0.0006) (n=16 PBS, n=17 IIIA4, 4 experiments). (E) Percentage of engraftment in spleen and bone marrow of LK63 mice treated with IIIA4 or PBS showed a significant reduction in spleen engraftment (P value. 0.0001) and a reduction in bone marrow engraftment (n=16 PBS, n=17 IIIA4, 4 experiments). (F) Spleen weight for IIIA4 and PBS treated Reh mice showed no significant differences between the two groups (n=15, 3 experiments). (G) Percentage of leukemic engraftment in spleen and bone marrow of Reh mice treated with IIIA4 or PBS was not significantly different (n=15, 3 experiments). Each dot on the graph D and E corresponds to one individual mouse. The data are presented as mean percentage ± SEM of spleen or bone marrow engraftment. An unpaired *t* test was performed for statistical analyses.

The data from LK63 and Reh leukemic models are consistent with a significant, direct anti-leukemic effect of IIIA4 on EphA3-positive LK63 cells. The lack of any effect on the EphA3-negative Reh cells is consistent with there being no effect on the stromal elements, which form the tumour microenvironment. Interestingly, apart from the effect on LK63 tumour growth and spleen and bone marrow engraftment, there appears to be a secondary effect on spread of the leukemic cells from bone marrow to the spleen. Even when animals were terminally ill and had bone marrow engraftment of almost 100% in both treated and un-treated animals the degree of splenic infiltration as indicated by both % engraftment and total spleen weight was dramatically less in treated animals. This disparity suggested an effect on tumour mobilisation and/or homing.

To further define the specificity of IIIA4 treatment, LK63 EphA3-knockdown (LK63/A3KD) and Reh EphA3-expressing (Reh/A3) cells were developed. The LK63/A3KD cells were developed by transducing LK63 cells with an EphA3 shRNA expressing lentivirus with a GFP-tag and selecting for low EphA3-expressing cells

using puromycin, followed by, sorting the low EphA3 and high GFP expressing cells. The established LK63/A3KD line showed EphA3 levels of <10% of parental control LK63 cells. Reh/A3 cells were derived by infecting Reh cells with EphA3-encoding lentiviral particles (GeneCopeia). Reh/A3 cells were selected using puromycin selection and then sorted for EphA3-positive cells with level of EphA3 expression comparable to LK63 cells (Figure 4.4A, B).

Engraftment of LK63/A3KD cells in NOD/SCID mice was somewhat slower when compared to control LK63 cells engraftment. Significantly smaller spleen size compared to normal LK63 xenograft spleen and significantly less bone marrow engraftment at the time of cull was observed in these mice. Comparable percentage of spleen engraftment was observed which could indicate significantly less engraftment in total spleen cells of LK63/A3KD considering that it has significantly smaller spleen compared to LK63 (Figure 4.4C, D).

More importantly in the LK63/A3KD xenografts, treatment with IIIA4 mAb did not significantly affect the spleen and bone marrow engraftment when compared to the PBS treated control group (Figure 4.4E, F). In contrast, when xenografts of Reh/A3 cells were treated with IIIA4 mAb there were a significant reduction in bone marrow engraftment compared to PBS treated control group. In Reh/A3 xenograft model similar to the Reh xenograft model there is a very low engraftment in the spleen however in this model a statistically significant reduction in spleen engraftment was observed. In the Reh/A3 xenograft model treatment with IIIA4 results in reduction in leukemic engraftment as opposed to Reh xenograft model where no therapeutic effect was observed in either spleen or bone marrow (Figure 4.4G, H).

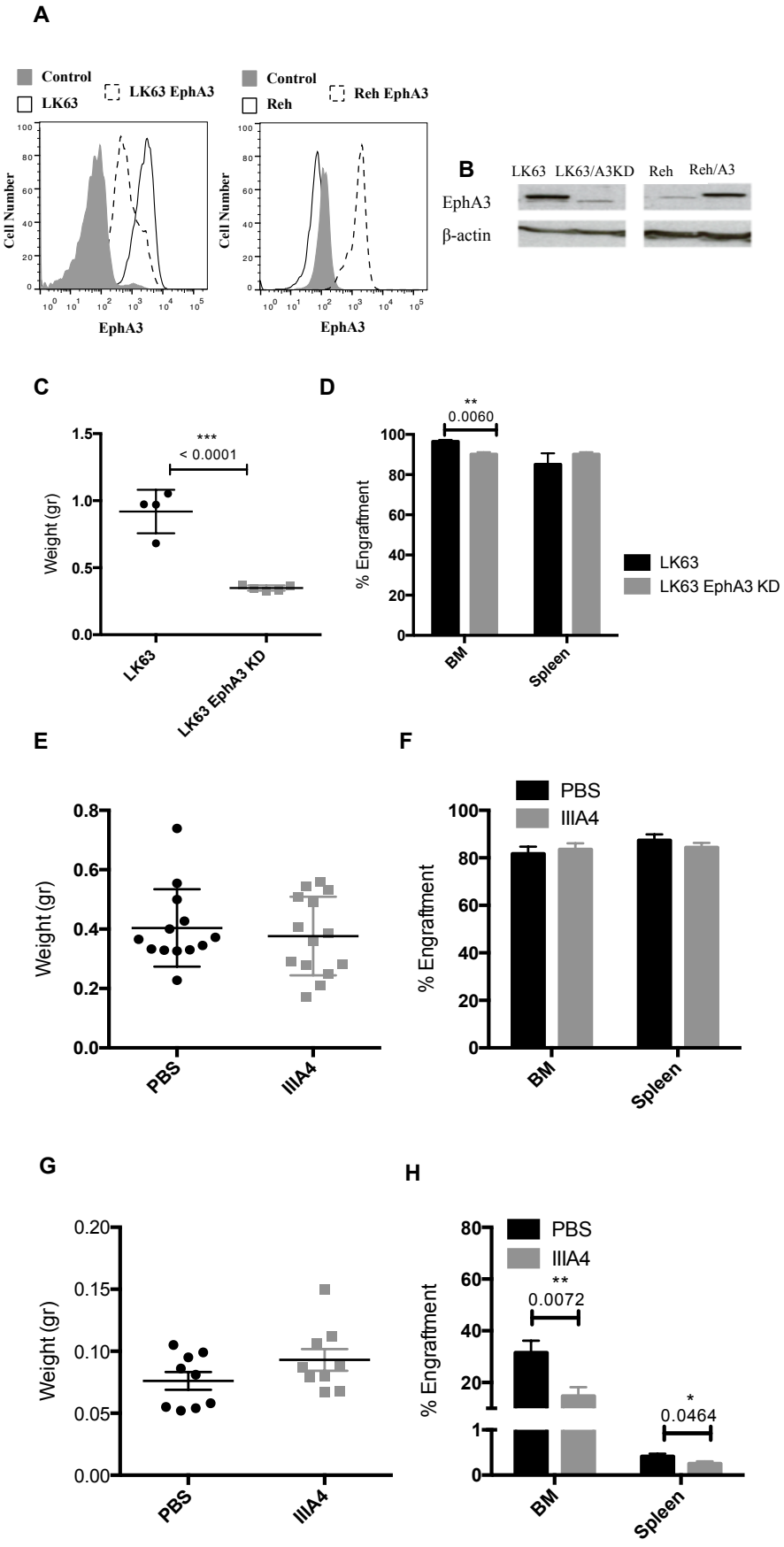
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**Figure 4.4. LK63/A3KD and Reh/A3 leukemic mice treated with PBS or IIIA4 mAb. (**A) Flow cytometric and analysis of LK63 and LK63/A3KD, Reh and Reh/A3 cells. (B) EphA3 and β-actin expression analysis determined using western blot analysis of LK63 and LK63/A3KD, Reh and Reh/A3 cells. (C) Comparison of spleen weight from LK63 and LK63/A3KD mice showed significantly smaller spleens in LK63/A3KD mice compared to EphA3 positive LK63 xenograft (P value. <0.0001) (n=4 PBS, n=5 IIIA4). (D) Percent engraftment in bone marrow of LK63 compared to LK63/A3KD mice showed significantly less engraftment in the bone marrow of LK63/A3KD xenograft (P value. 0.0060) (n=4 PBS, n=5 IIIA4) and comparable percent engraftment of spleen cells. (E) LK63/A3KD mice showed no significant differences in spleen weight between the IIIA4-treated and the PBS-treated control group (n=13 PBS, n=14 IIIA4, 3 experiments). (F) Percent engraftment in spleen and bone marrow of LK63/A3KD mice treated with IIIA4 or PBS showed no significant differences between the IIIA4 treated and PBS treated control group (n=4 PBS, n=5 IIIA4). (G) Reh/A3 mice showed no significant differences in spleen weight of the IIIA4-treated group compared to PBS-treated control mice. (H) Percent engraftment in spleen and bone marrow of Reh/A3 mice treated with IIIA4 or PBS showed a significant reduction in bone marrow (P value. 0072) and spleen (P value. 0.0464) engraftment in the IIIA4-treated group compared to PBS-treated control group. Each dot on the C, E and G plot corresponds to one individual mouse. The data are presented as mean percentage ± SEM of spleen or bone marrow engraftment. An unpaired *t* test was performed for statistical analyses.

To further investigate differences in engraftment of Reh and LK63 xenograft models, I transduced Reh and LK63 cells with a firefly luciferase containing lentivirus to isolate Reh/Luc and LK63/Luc cell lines. I used this method to track and image migration of LK63 and Reh cells in mice intravenously injected with $5\times10^6$ Reh/Luc or LK63/Luc cells. The mice were subsequently monitored twice on a weekly basis with Xenogen imaging and, as before, culled when they exhibited significant signs of illness (Figure 4.5). These mice were treated with 100 μg of IIIA4 or PBS on alternate days. Consistent with the previous results, the LK63 mice treated with IIIA4 showed similar bone marrow engraftment to PBS-treated mice at early stages of leukaemia progression; however, in the later stages of leukaemia progression minimal splenic engraftment was observed in the IIIA4-treated group compared to high levels of engraftment in the PBS-treated controls (Figure 4.5A). An interesting observation was the apparent decrease in signal from bone in mice with massively enlarged spleens. This appears to be an artefact as the bone marrow analysis showed progressive infiltration. A possible explanation is that there is competition for luciferin substrate whereby the massive splenic involvement "starves" other tissues of substrate. In the Reh model of leukaemia the Xenogen image analysis was consistent with previous

engraftment results in which no splenic engraftment was observed in both PBS- and IIIA4-treated mice and the bone marrow engraftment was comparable or increased in IIIA4-treated mice compared to the PBS-treated group (Figure 4.5B).

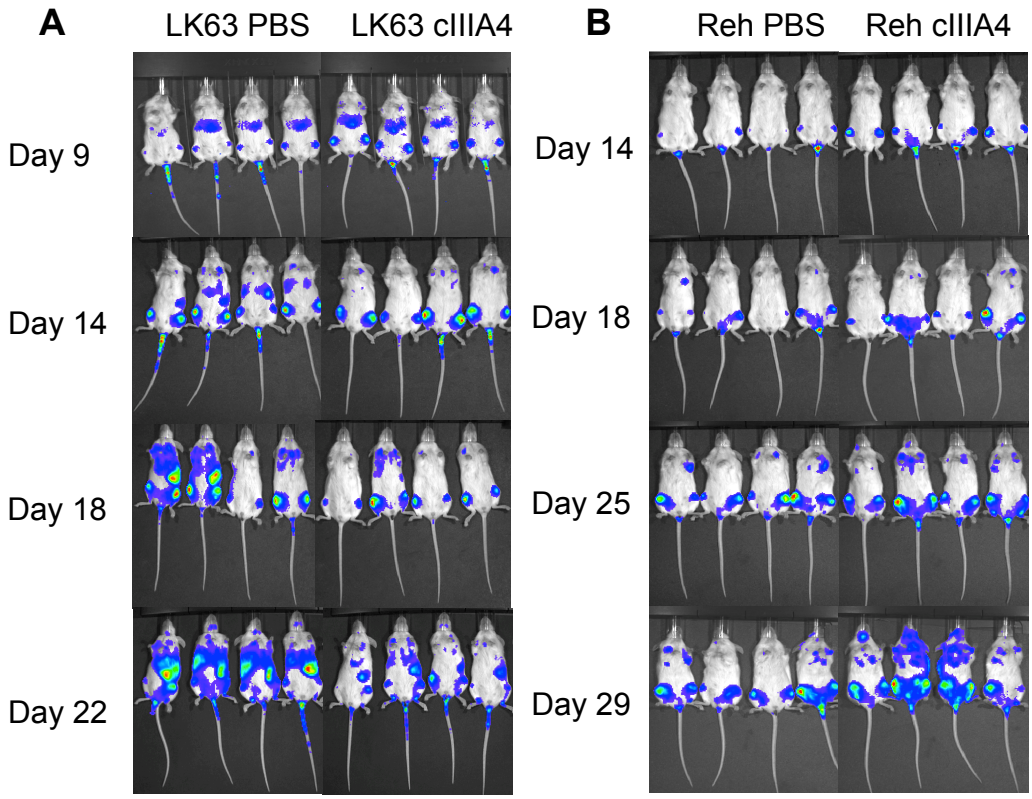



**Figure 4.5. Xenogen images of Reh and LK63 leukemic mice**. (A) LK63 leukemic engraftment model treated with PBS or IIIA4 mAb on alternate days and representative images from day 9, 14, 18 and 22. On day 9 there was minimal and similar engraftment in the bone marrow of PBS- and IIIA4-treated mice. On day 14 PBS-treated mice have minimal engraftment in the spleen and IIIA4-treated group showed no spleen engraftment. On day 18 and 22 PBS-treated mice have significantly higher engraftment in the spleen compared to IIIA4-treated group. (B) Reh leukemic engraftment model treated with PBS or IIIA4 mAb on alternate days and representative images from day 14, 18, 25 and 29. The mice showed similar engraftment on day 14, 18 and 25; however, on day 29 the mice treated with IIIA4 showed higher bone marrow engraftment compared to PBS treated group.

The previous results had suggested that initial engraftment only occurred in bone marrow and that spread to spleen and other sites was a secondary event. In an attempt to confirm this, I developed an intrafemoral model of leukaemia in which $2\times10^5$ Reh/Luc or LK63/Luc cells were directly injected into the right femur and mice were monitored for pattern of leukemic spread, using Xenogen imaging, and signs of illness. Similar to previous models, these mice were also treated with 100 µg of IIIA4

mAb or PBS on alternate days until the mice were euthanized due to progression of disease (Figure 4.6).

The LK63 intrafemoral mouse model showed that the LK63 cells initially engrafted at the injection site in the right femoral bone marrow and then engrafted to the other bones before the leukemic cells spread to the spleen. This was not observed in the Reh model of engraftment in which the cells grow in the injected bone marrow and then move to the other bones and, as with intravenously injected cells, did not significantly engraft in the spleen (Figure 4.6). Thus, the pattern of spread observed in this model recapitulated the patterns observed in animals injected intravenously. For LK63, in particular, this confirmed that leukemic cells preferentially engraft and grow in the bone marrow and only once the disease is well-advanced spread to other sites, notably the spleen. The marked reduction in splenic engraftment in IIIA4-treated animals again suggests that infiltration of the spleen may depend, at least in part, on EphA3.

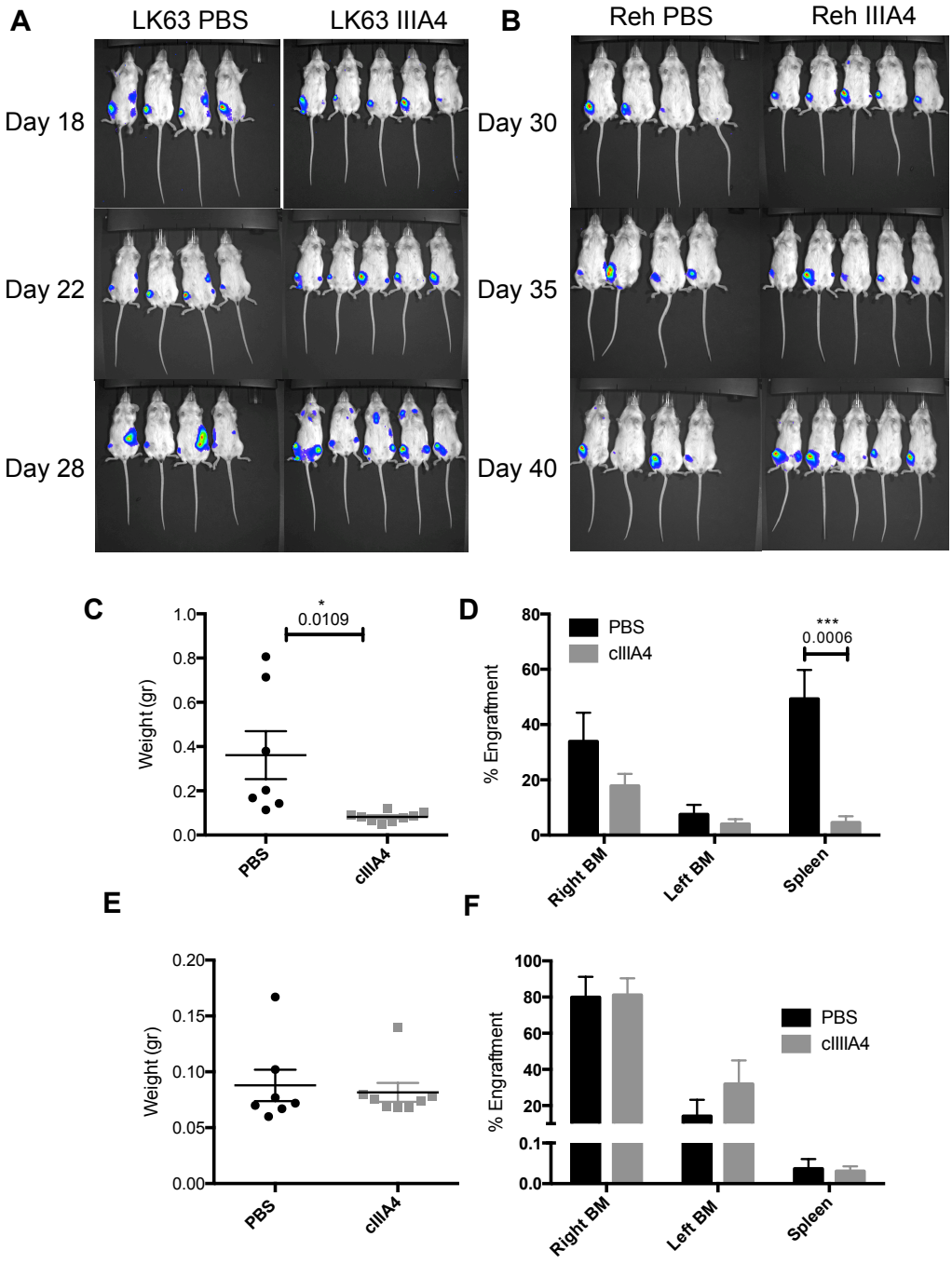
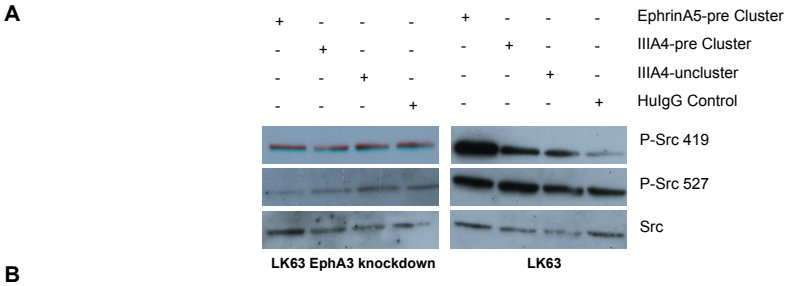



**Figure 4.6. Intrafemoral model of Reh and LK63 Leukemic mice.** (A) LK63 intrafemoral model treated with PBS and IIIA4 representative images from day 18, 22 and 28 showed initial right bone marrow engraftment followed by left bone marrow engraftment and spleen engraftment in the PBS treated model and displayed a much slower disease progression in the IIIA4 treated group. (B) Reh intrafemoral model treated with PBS and IIIA4 imaged on days 30, 35 and 40 showed initial engraftment in the right femoral bone marrow followed by significant engraftment in the left femoral bone marrow with no splenic engraftment up to day 40. (C) Spleen weight for LK63 intrafemoral model showed significantly smaller spleen (P value. 0.019) in the IIIA4-treated group at the time of cull (n=7 PBS, n=9 IIIA4, 2 experiments) (D) Percent engraftment in the right bone and left bone

marrow of LK63 intrafemoral model treated with IIIA4 was less than PBS-treated mice. Percent engraftment in the spleen of LK63 intrafemoral model treated with IIIA4 was significantly less (P value: 0.0006) compared to the PBS-treated group (n=7 PBS, n=9 IIIA4, 2 experiments). (E) Spleen weight for Reh intrafemoral model showed no differences between PBS and IIIA4 treated mice (n=7 PBS, n=9 IIIA4, 2 experiments). (F) Percent engraftment in the right bone marrow of the Reh intrafemoral model was similar between IIIA4- and PBS-treated groups. Percent engraftment in the left bone marrow was higher in the IIIA4-treated compared to the PBS-treated mice. Percent engraftment in the spleen of the Reh intrafemoral model treated with IIIA4 was minimal but less compared to PBS treated group (n=7 PBS, n=8 IIIA4, 2 experiments). Each dot on the plot corresponds to one individual mouse. The data are presented as mean percentage ± SEM of spleen or bone marrow engraftment. An unpaired *t* test was performed for statistical analyses.

### 4.3.4 The effect ephrinA5 or IIIA4 antibody induced stimulation on LK63 and Reh cells

In seeking to define how the antibody treatment may affect LK63 cell migration and homing, LK63 cells were incubated with either PBS, pre-clustered huIgG, IIIA4, pre-clustered IIIA4 or pre-clustered ephrinA5-Fc, a form of the high affinity ligand which induces receptor clustering and activation. I examined the effect of these stimuli on several pathways downstream of Eph receptors including Akt, the MAP kinases Erk1/2 and Src. In LK63 cells, treatment with IIIA4, pre-clustered IIIA4 and pre-clustered ephrinA5-Fc resulted in increased phosphorylation of the activation loop of Src on tyrosine 419 with no significant effect on tyrosine phosphorylation of the auto-inhibitory 527 site (Figure 4.7A). No effect was observed on Akt or Erk phosphorylation. More importantly, no activation of any of the above pathways, including Src, was observed in LK63/A3KD cells. I also looked at the effect of EphA3 activation on cell morphology and shape in LK63 and LK63/A3KD using live cell imaging over 20 minutes with one frame per 10 seconds. This showed increased cell rounding and membrane blebbing in response to activation with IIIA4, pre-clustered IIIA4 or pre-clustered ephrinA5-Fc; this effect was less pronounced in LK63/A3KD cells (Figure 4.7B-C).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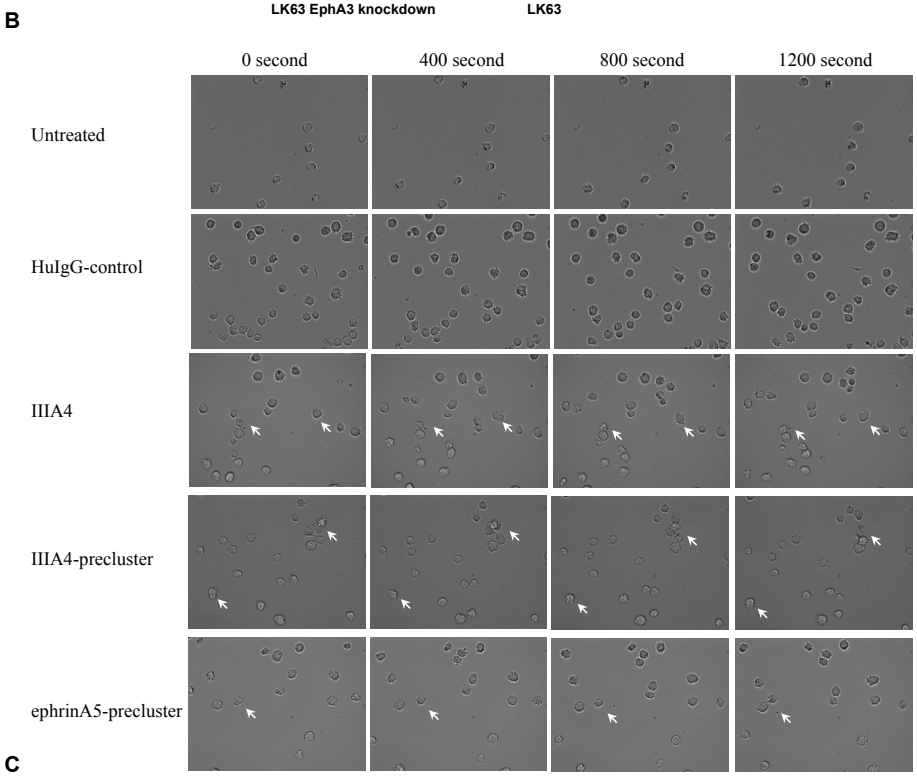
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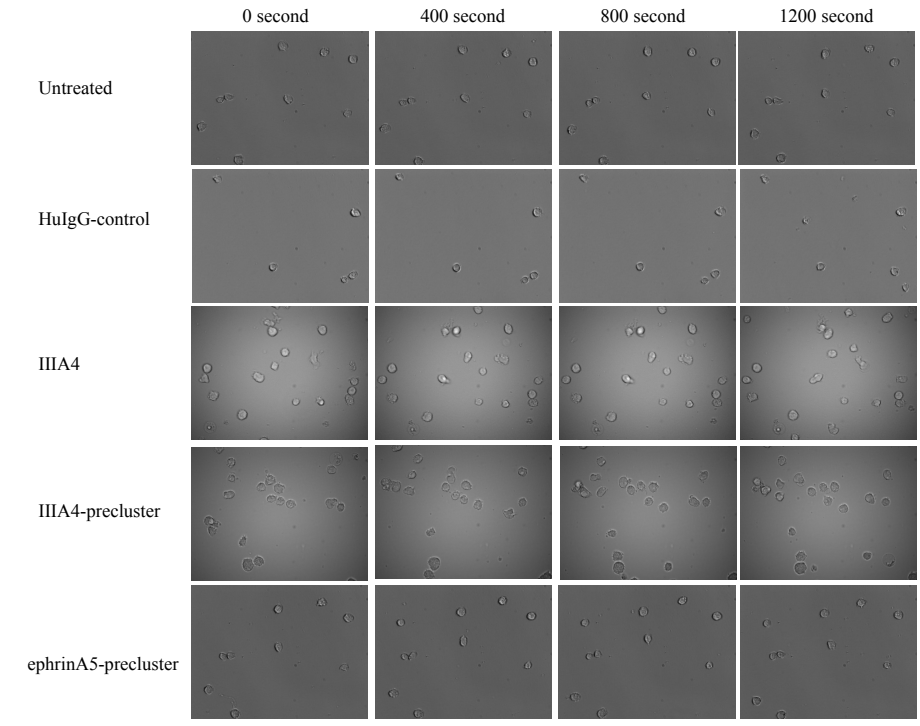
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**Figure 4.7. The effect of EphA3 activation on downstream signalling and cell morphology in LK63 and LK63/A3KD cells.** (A) Increase phosphorylation of Src on tyrosine 419 was observed in LK63 cells upon activation with IIIA4, pre-clustered IIIA4 or pre-clustered ephrinA5-Fc compared to the PBS control. Stimulation had no effect on phosphorylation of Src on tyrosine 527. In the LK63/A3KD phophorylation of Src on tyrosines 419 and 527 was unaffected by these stimuli. (B) Representative images from LK63 cells upon activation with IIIA4, pre-clustered IIIA4 or pre-clustered ephrinA5-Fc. The images showed membrane blebbing and cytoskeleton contraction upon EphA3 activation. The arrows point to the cells with membrane blebbing or cytoskeleton contractions. (c) Representative images from LK63/A3KD cells upon activation with IIIA4 pre-clustered IIIA4 or pre-clustered ephrinA5-Fc showing minimal changes in cell shape and morphology.

### 4.3.5 Radiolabelled IIIA4 Mab antibody treatment

#### 4.3.5.1 Immunoreactivity of radiolabelled IIIA4

$^{213}$Bi-IIIA4 and control $^{213}$Bi-labelled antibodies were prepared at respective specific activities of 109 mCi/mg and 167 mCi/mg for *in vitro* and *in vivo* characterisation. Assessment of radiochemical purity by Instant Thin Layer Chromatography (ITLC) consistently demonstrated >99% $^{213}$Bi- bound to IIIA4 and 97.7% for the isotype control. Immunoreactivity of $^{213}$Bi-IIIA4 for LK63 cells was determined by the single point cell binding assay and was 38.7%.

#### 4.3.5.2 Biodistribution

The biodistribution results for $^{213}$Bi-radiolabeled IIIA4 and isotype control treatment in LK63 leukemic NOD/SCID mice are presented in Figure 4.8. The biodistribution studies indicate that $^{213}$Bi-IIIA4 localised to the expected areas of leukaemia involvement, including blood, spleen, lung and liver, within 15 minutes of injection and were maintained over the 3 hours of the experiment. Peak uptake of 20.8 ± 3.9 %ID/g and 20.9 ± 3.8 %ID/g were observed at 2 hours in the liver and spleen, respectively (Fig 4.8A). Minimal uptake (0.2–6 %ID/g) was observed in other normal tissues at all time points (Fig 4.8A). The specificity of the $^{213}$Bi-IIIA4 for EphA3-positive leukaemia was confirmed by the low levels of tissue localisation observed with the isotype control antibody (Figure 4.8B).

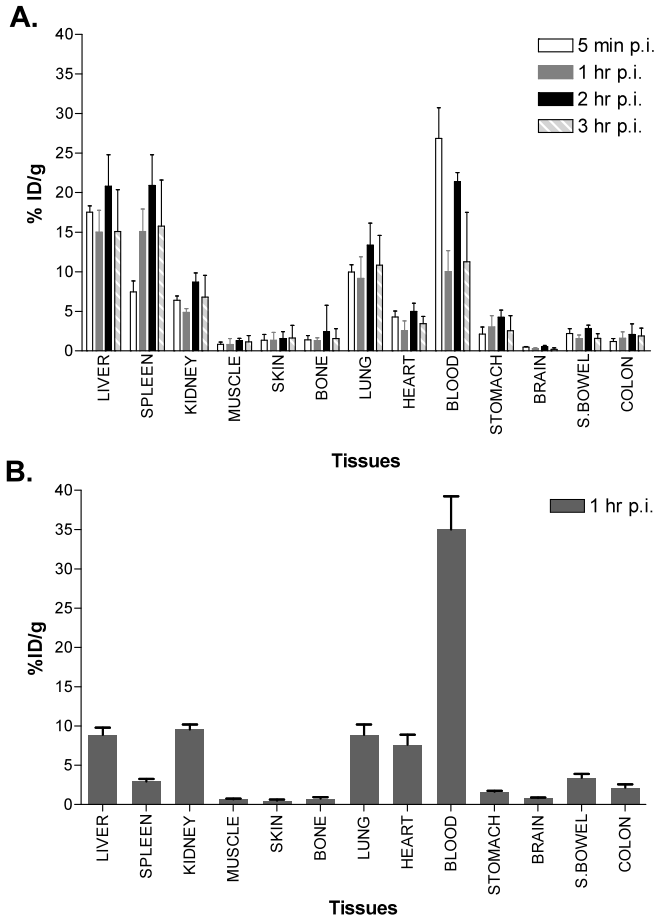


**Figure 4.8. Biodistribution of $^{213}$Bi-labeled IIIA4 and isotype control antibodies.** (A) $^{213}$Bi-IIIA4 biodistribution in LK63 leukemic NOD/SCID mice at 5 minutes, 1 hour, 2 hours and 3 hours after treatment. (B) Distribution of $^{213}$Bi-isotype controls in NOD/SCID mice bearing LK63 cells at 1 hour after treatment. (n=4). Results of the biodistribution are presented for each tissue as mean percent of injected dose per gram of tissue (%ID/g) values ± SD.

### 4.3.5.3 Efficacy of $^{213}$Bi-IIIA4 therapy

The effect of a single dose of $^{213}$Bi-IIIA4 radioimmunotherapy in the LK63 xenograft at day 6 post LK63 leukemic cell injection showed no significant difference in the median survival between the saline-treated group, $^{213}$Bi-labeled isotype control and $^{213}$Bi-IIIA4 at 12.5 or 25 mCi dose levels (Figure 4.9A).

The effect of four doses of $^{213}$Bi-IIIA4 on animal survival of leukaemia engrafted NOD/SCID mice is presented in Figure 4.9. Following engraftment on Day 0, the median survival for the vehicle control group was 21 days. Multi-dose treatment with unlabelled IIIA4 or 12.5 mCi $^{213}$Bi-isotype control commencing on Day 2 caused a

slight increase in median survival to 24 days. Radioimmunotherapy with $^{213}$Bi-IIIA4 significantly increased median survival in a dose dependant manner compared to IIIA4 protein alone, with a median survival of 26.5 days at the 12.5 mCi repeat dose and 41.5 days at the 25 mCi repeat dose. The specificity of the IIIA4 radioimmunotherapy is indicated by the significantly shorter survival of the corresponding arms of $^{213}$Bi-isotype control antibody-treated groups at both the 12.5 mCi and 25 mCi repeated dose levels (Figure 4.9B).

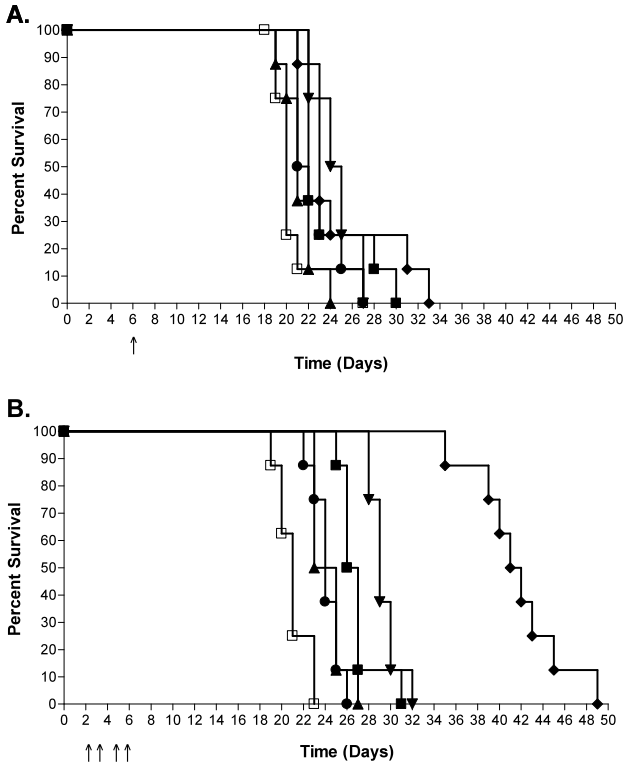


**Figure 4.9. Survival curve in response to treatment with $^{213}$Bi-IIIA4.** (A) Single dose $^{213}$Bi-IIIA4 radioimmunotherapy treatment following engraftment with LK63 leukemic cells on Day 0 showed no significant differences between the control groups (saline, 180 mg IIIA4, $^{213}$Bi-isotype control at 12.5 and 25 mCi) and the $^{213}$Bi-IIIA4-treated (12.5 mCi and 25 mCi) groups. (B) Multi-dose $^{213}$Bi-IIIA4 radioimmunotherapy following engraftment with LK63 leukaemia cells on Day 0 showed significant increase in survival of IIIA4 or 12.5 mCi $^{213}$Bi-isotype control (P value. 0.001) compared to saline control. Significant increase in survival of 12.5 mCi $^{213}$Bi-IIIA4 (P Value. 0.01) and 25 mCi $^{213}$Bi-IIIA4 (P value. <0.0001) was observed compared to IIIA4 control group. Survival of the 12.5 mCi $^{213}$Bi-IIIA4 compared to 12.5 mCi $^{213}$Bi-isotype was significantly improved (P value. 0.0011). Survival of the 25 mCi $^{213}$Bi-IIIA4 compared to 25 mCi $^{213}$Bi-isotype was significantly improved (P value. 0.0001). (n=8). Days of injection of with saline (□), 180 mg mAb IIIA4 (▲), $^{213}$Bi-isotype controls at 12.5 mCi (●), or 25 mCi (▼) or $^{213}$Bi-IIIA4 at 12.5 mCi (■), or 25 mCi (◆) are indicated by arrow.

## 4.4 Discussion

Given the expression of EphA3 in many different human cancers including leukaemia, I explored the expression and function of EphA3 and the possibility of using it as a therapeutic target in the treatment of leukaemia. Of the tested EphA antigens, I found EphA3 to be the most prominent Eph expressed on a panel of leukemic patient samples and cell lines. Therefore I have investigated therapeutic targeting of EphA3 in two pre-B ALL cell lines, the EphA3-positive LK63 and the EphA3-negative Reh cell lines.

In these models, LK63 cells preferentially grow in the bone marrow followed by engraftment in the spleen and peripheral blood; however, Reh engraftment was observed to be largely confined to the bone narrow with minimal splenic engraftment. Administration of the IIIA4 anti-EphA3 mAb to LK63 xenograft resulted in inhibition of tumour growth and marked delay in spread from bone marrow to the spleen and other organs and increased in the latency of the disease. However, no therapeutic effect was observed in the Reh xenograft model suggesting that the effect of IIIA4 mAb treatment is primarily directed against the leukemic cells expressing EphA3 with little or no effect on host cells.

To further confirm that EphA3 bearing leukemic cells were the direct target of the IIIA4 treatment, LK63 EphA3-knockdown (LK63/A3KD) and Reh EphA3-expressing (Reh/A3) cell lines were developed. Similar to the lack of antibody treatment effect on Reh xenograft group, treatment of LK63/A3KD xenograft with IIIA4 showed no significant changes in the spleen and bone marrow engraftment or spleen weight. However, treating Reh/A3 mouse with IIIA4 showed significant reduction in bone marrow and spleen engraftment similar to the therapeutic effect observed in LK63 xenograft. These results confirm that the effect of EphA3 mAb treatment is directed against the leukemic cells rather than the stromal and vascular elements.

To understand the mechanism leading to therapeutic effect of IIIA4 on EphA3-expressing leukemic cells, I looked at the effect of EphA3 activation on the LK63 and LK63/A3KD cells. My analysis showed elevated Src kinase activity upon stimulation with either IIIA4 or ephrinA5-Fc. Src kinases have been implicated in Eph-mediated signalling in other systems, and rather than a growth promoting effect, its activation

appears to target adhesive mechanisms, particularly integrins, and cell motility functions [197-199]. There is abundant evidence in other cells that the latter functions of Eph proteins are mediated by activation of RhoA leading to actin depolymerisation and inactivation of Rac and Cdc42 leading to retraction of cell processes and decrease in cell migration [52,64,200]. My preliminary analysis of cell behaviour in response to antibody binding shows an increase in cell rounding and membrane blebbing as it was previously demonstrated in LK63 cells in response to ephrin stimulation [68]. Future studies are needed to fully elucidate the role of Src signalling in initiating the cell shape and movement changes induced by antibody binding which may underpin the observed anti-leukemic effect.

The other aspect of these studies was to explore if EphA3 could be used as a targeting agent, particularly given its low level expression in normal tissues. In glioblastoma treatment with radiolabelled IIIA4 antibody showed a great anti-tumour effect in EphA3-positive glioblastoma tumours [59]. In the LK63 model an alpha particle emitting isotope was used to minimise the off target effects on EphA3-negative cells. Consistent with the Day et al. (2013) finding, an impressive anti-tumour effect on EphA3 positive leukemic model was delivered by the alpha particle emitting bismuth-213 isotope directly linked to the IIIA4 antibody. These results strongly suggest that the direct inhibitory effect of IIIA4 therapy on EphA3 positive leukaemias might be further enhanced by payloading with an isotope or with cytotoxic drugs, as has been demonstrated for the anti-HER2 antibody trastuzumab in breast cancer [201].

# Chapter 5 . GENERAL DISCUSSIONS AND FUTURE DIRECTIONS

I started out with the knowledge that various members of Eph/ephrin family of proteins are expressed on haematopoietic cells and leukemic tumours [75]. However, there were limited data on the functional effect of Eph family of RTKs in normal haematopoiesis and leukaemia. In this thesis I aimed to explore the potential functional effects of several members of the EphA subfamily of Eph RTKs. In particular, I explored the roles of EphA1, EphA2 and EphA7 in haematopoiesis by utilising the available knockout mice. I have also investigated the role of EphA2 and EphA3 in leukaemogenesis. My lab had highly specific monoclonal antibodies to all of these Eph proteins, providing the essential tools for exploring the targeting of these proteins in the treatment of haematological malignancies.

In Chapter 2, I examined the functional role of EphA1, EphA2 and EphA7 in normal haematopoiesis by using the EphA1, EphA2 and EphA7 null mice in comparison to congenic littermates. This study demonstrated that loss of EphA1, EphA2 or EphA7 does not significantly disrupt normal haematopoiesis. In essence, these mice have a normal steady haematopoietic system. Expression of other members of Eph family have been reported on haematopoietic cells which could be an indication that other members of this family of RTKs can compensate for the function in the absence of one member. For example, Ting et al. (2011) reported expression of EphA3 and EphA5, both of which are functionally similar to EphA2 and EphA7, on haematopoietic progenitor cells [123]. The necessary experiments to explore redundancy were not feasible within the scope of this project. Therefore, in future it will be important to investigate the potential redundancy of Eph receptors in haematopoiesis by studying the functional effect of Eph blockade using broad spectrum inhibitors or examining mice with multiple Eph deletions on hematopoietic system. Another alternative approach would be to pathologically "stress" the haemopoietic system, for example with infection, and see whether the response to this challenge in knockout mice is normal.

The study presented in chapter 3 initially examined expression of Eph genes in two models of the leukaemia, the MLL-AF9 model of acute myeloid leukaemia and the BCR-ABL model of chronic myeloid leukaemia. Aberrant expression of Eph genes specifically EphA2 and EphA7 were only significantly present in the MLL-AF9 model, a finding foreshadowed by previous studies [113]. Given the availability of well-

characterised reagents for EphA2, I focused on the potential role of EphA2 in MLL-AF9 leukaemia. These studies showed that lack of EphA2 expression did not significantly alter the leukaemogenic process initiated by the MLL-AF9 oncogene. However, as expression of other members of EphA subfamily was found on MLL-AF9 leukemic cells, this may reflect again the redundancy of function such that other members of Eph family proteins compensate for loss of EphA2. This could be further explored once knockout animals with several EphA deletions are available. A small survey of human leukaemias suggested that EphA1, EphA2, EphA3 and EphA7 are variably expressed on MLL-driven leukaemias and thus combination knockouts of these genes could be prioritised.

I then examined whether EphA2 could serve as a therapy target in MLL-AF9 leukaemia using the IF7 monoclonal antibody, which specifically binds to human and mouse EphA2 protein. There was, however, no direct anti-tumour effect delivered upon treatment of MLL-AF9 mice with EphA2 monoclonal antibody (IF7 mAb). This monoclonal antibody is an IgG1 isotype antibody, thus it does not have any significant Fc effector function. Therefore a different isotypic form of EphA2 mAb might provide some functional effect in the treatment of the EphA2 positive MLL-AF9 leukaemia. This could be verified through the development of new antibodies or by engineering the IF7 V regions into, for example, a mouse IgG2a backbone.

Next, I investigated if the antibody could be used to specifically target a cytotoxic payload to MLL-AF9 leukemic cells. I showed that IF7 radiolabelled with lutetium was capable of significantly delaying the course of MLL-AF9 leukaemias. Given the highly aggressive nature and therapy refractoriness of this model of leukaemia, this was an exciting finding, which needs further exploration. The experiments presented were performed with mice in the late stages of disease and with only two doses of radiolabelled antibody treatment. Thus, further experiments are needed to determine if treating at an earlier stage when blasts first appear in the blood, with more sophisticated dosing regimens, perhaps combined with chemotherapy, could induce a more profound effect. The EphA2 targeting also provides proof of principle that other Eph monoclonal antibodies, including EphA3 or EphA7, could be used in a similar fashion. In adults MLL-driven leukaemias represent a minority of acute leukaemias but in childhood, particularly in infants, they commonly cause acute lymphoblastic

leukaemia with poor prognosis. Thus, antibodies that target these leukaemias may well have clinical usefulness, particularly in the paediatric setting [202].

In chapter 4, the anti-leukemic effect of an antibody to EphA3, IIIA4 was examined. The EphA3 antibody was shown to exert a direct anti-leukemic effect in the LK63 pre-B ALL model. By further testing of the EphA3-positive and the EphA3-negative xenograft models, I was able to show that this was a direct effect on the leukemic cells, with no evidence of an effect on normal host cells. *In vitro* studies had previously shown that this antibody had no effect on proliferation or cell survival but it clearly exerted an anti-tumour effect *in vivo*. Notably, treatment of the LK63 model with IIIA4 mAb had shown effects on tumour spread. This finding indicated that the antibody was able to directly alter spread from the bone marrow to other sites and suggested an effect on adhesion and migration. In an attempt to understand the effect of IIIA4 treatment on adhesion and migration of EphA3 positive leukemic cells, I showed that both ephrin ligand and IIIA4 could induce autophosphorylation of the Src kinase. Src kinase has been previously linked with a pathway that disrupts the cytoskeleton such that cells became less adhesive and motile [197]. Consistent with effects on this pathway, I found that antibody treatment induced cells rounding and membrane blebbing, cellular changes, which had been reported previously in response to ephrin-stimulation [68]. In future studies it will be important to analyse signalling pathways downstream and upstream of Src signalling pathway, including FAK, Rho family GTPases and paxillin. This will provide further insight into the mechanism involved in the anti-leukemic effect of the IIIA4 mAb treatment.

The overall effect of IIIA4 mAb treatment in the LK63 model was a modest prolongation of survival suggesting that antibody alone might not be highly effective as a therapy. However, these models were depended on xenografts, which only formed in highly immunocompromised mice and thus there was no antibody-mediated immune response that might synergise with the direct effect. In this regard, Kalobios has engineered a humaneered form of the antibody and shown that this elicits strong antibody-dependent cell cytotoxicity [203]. This form of EphA3 antibody, KB004, is now in clinical trial. In the xenograft models, as with the studies of the EphA2 mAb in the MLL-AF9 model, a much more potent anti-tumour effect was

observed when IIIA4 was used to deliver a cytotoxic payload to the leukemic cells providing a further possible therapeutic approach for EphA3-positive leukaemias.

Overall, in this thesis I showed that individual members of the Eph family are not essential for haematopoiesis and leukaemogenesis. However, I have shown that the Eph RTK family, in particular EphA2 and EphA3, represents a new group of potential therapy targets in haematological malignancies. Targeting of these proteins with monoclonal antibodies provides a highly specific means of delivering a cytotoxic payload to the tumour cells. I have thus far only explored delivery of radioactive isotopes but in principle this approach could also be used to deliver cytotoxic drugs, an approach already being used for other targets [157].

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# APPENDIX

Leukemia

Charmsaz and Boyd, J Leuk 2013, 1:1
http://dx.doi.org/10.4172/jlu.1000107

Review Article Open Access

# Expression and Function of the Eph Receptor Family in Leukemia and Hematopoietic Malignancies: Prospects for Targeted Therapies

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**Abstract**

There has been considerable interest in recent years in the development of therapies, which target Eph receptors or their ephrin ligands. The Eph receptor tyrosine kinases and their membrane bound ephrin ligands are cell surface molecules involved in many biological functions and cell behaviors during embryogenesis and in adult life. However, they are also expressed in an aberrant fashion on many tumors and are re-expressed on normal cells in non-malignant pathological states. Some of the eph/ephrins including EphA7 and EphA1 protein are thought to function as tumor suppressors in particular cancers. In tumors where Eph/ephrin proteins are expressed at high levels, being expressed on the cell surface these proteins are readily accessible to antibody-mediated therapies several of which are in advanced pre-clinical or early clinical evaluation, including antibodies specific for EphA2, EphA3, and EphB4 which are expressed on many different tumors. We will review the general features of the Eph/ephrin system and discuss the role of this system in normal hematopoiesis before focusing on the role of Eph proteins in leukemia and other hematological malignancies and possible avenues for therapy.

**Keywords:** Eph receptor; Ephrin; Receptor Tyrosine Kinase; Hematopoiesis; Hematopoietic Stem Cells; Leukemia

## Classification, Structure and Binding of Eph/ephrin

The Eph receptors and their membrane bound ephrin ligands represent the largest family of receptor tyrosine kinases (RTK's). The first member, EphA1, was originally termed Eph, as it was first identified in an Erythropoietin-Producing Hepatocellular (Eph Nomenclature committee, 1997) carcinoma cell line [1]. Fourteen members of the Eph family of RTK have been identified in mammals, which are divided into two groups, the EphA and EphB family. This is based on their sequence homology, ligand specificity and structural features. In mammals there are nine members of the EphA subgroup (EphA1-8 and EphA10) and five EphB receptors (EphB1-4 and EphB6) [2,3].

Eph receptors are type I transmembrane proteins composed of extracellular region including a ligand binding domain with two distinct ligand-binding interfaces (N terminal β jelly roll domain) determining the ephrin binding, and a cysteine-rich region containing an epidermal growth factor (EGF)-like motif that is involved in receptor dimerization followed by two fibronectin type-III domains [4,5]. The intracellular region comprised of a conserved juxtamembrane domain, a tyrosine kinase domain and a sterile alpha motif (SAM) domain, the latter having a potential role in receptor clustering [6,7]. Most of the Eph receptors, possess a C-terminal PDZ (Postsynaptic density protein, Disc large, Zona occludens tight junction protein) binding motif that is involved in signaling at a sub-cellular level and in the assembly of large molecular complexes [3,5,8] (Figure 1a).

The ephrin ligands of Eph receptors are also membrane bound proteins and like the Ephs they are divided into two groups based on their structural features and preferential binding to either EphA or EphB receptors. The two classes of ephrin ligands are the A-class (ephrinA1-5) and B-class (ephrinB1-3) [3]. The two classes of the ephrin ligands have homology at their N-terminus region while their C-terminal amino acid sequence differs, the ephrin-A ligands that are bound to the plasma membrane by a glycosylphosphatidylinositol (GPI)-linker and the ephrin-B ligands, which possess a transmembrane–spanning region and a highly conserved cytoplasmic tail with a number of highly conserved tyrosine residues and a PDZ-binding motif. The conserved tyrosine residues have been shown to serve as a docking site for proteins, which mediate downstream ephrin signaling [2,9] (Figure 1b).

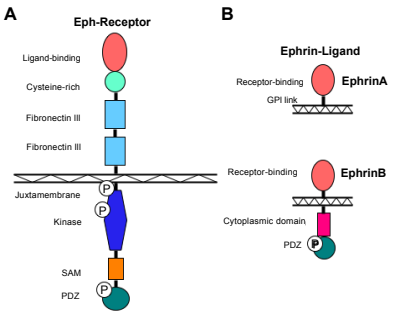


**Figure 1:** Schematic representation of Eph receptors and ephrin ligand structure. (A) The structure of the Eph receptor. (B) The structure of the ephrin-A and ephrin-B ligand.

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**Received** February 23, 2013; **Accepted** April 01, 2013; **Published** April 03, 2013

**Citation:** Charmsaz S, Boyd AW (2013) Expression and Function of the Eph Receptor Family in Leukemia and Hematopoietic Malignancies: Prospects for Targeted Therapies. J Leuk 1: 107. doi:10.4172/jlu.1000107

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Generally, the EphA receptors will preferentially bind to members of the ephrin-A ligands (ephrinA1-5) while the EphB receptors will preferentially bind to the ephrin-B ligands (ephrinB1-3) [3,10]. There is a high level of binding promiscuity between the Eph receptors and the ephrin ligands, however for an individual Eph receptor there is a distinct order of affinity of ephrin interactions with affinity constants ranging from 5-500 nM [11]. For instance the EphA1 receptor binds with a high affinity to ephrin-A1 and a lower affinity to other members of the ephrin-A family, including ephrin-A3 and ephrin-A4, and shows essentially no binding to ephrin-A5 [12]. Similarly EphA3 and EphB4 have a higher affinity for ephrin-A5 and ephrin-B2 respectively than for other members of the ephrin family [11,13]. High affinity interaction is also possible between the classes, for example EphA4 binds to both ephrin-B and -A ligands and some of its most important functions depend on interaction with ephrin-B3. Another example is EphB2, which binds to ephrin-A5 as well as ephrin-B ligands [14,15] (Figure 2).

## Eph/ephrin Activation and Signaling

Eph/ephrin interactions have the capacity to initiate bidirectional signaling. In other words both Ephs and ephrins can act as a ligand as well as a receptor and they both have the ability to initiate signaling. Signaling initiated by the Eph receptor is referred to as forward signaling whereas signaling initiated by the ephrin ligand is termed reverse signaling.

Eph receptor activation occurs following the physical association of the receptor and an ephrin ligand on adjacent cell surfaces mediated by the high affinity binding site (dimerization), this is followed by interaction with a second Eph/ephrin complex, mediated by separate low affinity (tetramerization) binding site to create a hetero-tetrameric complex [16]. The tetramers are then assembled into higher order clusters, which appear to be required for effective forward signaling [17,18]. The clustering of Eph receptors results in ligand-dependent auto-phosphorylation of several tyrosine residues within the cytoplasmic kinase domain and juxtamembrane region of the receptor, which serve as docking sites for downstream signaling proteins including the small GTPases of the Rho and Ras family, focal adhesion kinase (FAK), the Janus kinase/signal transducers and activators of transcription (Jak/Stat) and phosphatidylinositol 3-kinase (P13K) pathways [19].

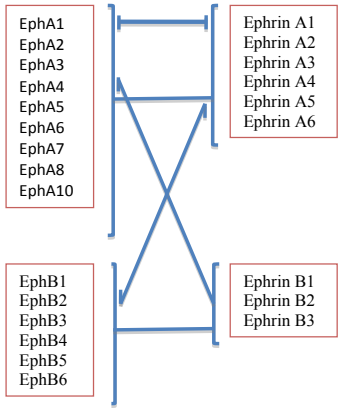


**Figure 2:** Schematic of Eph receptor and ephrin binding preference.

Eph receptor activation influence cell shape and motility through the regulation of the Rho GTPases, including RhoA, Cdc42, Rac and Ras, via interactions with specific Rho GTPase activating (Rho GAP) and exchange (Rho GEF) factors. The interaction with individual Rho GTPases, mediated via direct binding to the cytoplasmic domain of Eph proteins of Rho GAP and Rho GEF or their recruitment via adaptor proteins, thereby mediating different effects on actin dynamics and cell process formation thus regulating cell shape and movement. For example, activation of EphA4 receptor results in recruitment of ephexin (Rho GEF), leading to activation of RhoA, and α-chimaerin (Rho GAP), leading to inactivation of Rac, jointly resulting in actin depolymerization and retraction of cell processes [15]. Activation through EphB plays a role in actin filament extension, morphogenesis and maturation of dendritic spines. Ras family of GTPases (H- and R- Ras) are also activated through Eph receptors however, unlike the Rho GTPases, the majority of Eph receptors negatively regulate the Ras/Mitogen-activated protein kinase (Ras/MAPK) pathway, with activation normally resulting in regulation of proliferation and migration [15].

Eph receptors are also important in mediating a number of other molecules involved in cell migration and adhesion due to their ability to regulate signal transduction molecules including integrin signaling pathway elements paxillin, FAK, P130 (Cas) and integrins themselves. The effect of Eph signaling on integrins is complex as they can mediate either promoting or suppressing effects. FAK is important in mediating integrin signaling and Eph receptors can down-regulate this pathway [20].

The Jak/Stat pathway, involved in cell growth and viability, is regulated by EphA receptor activation [21]. EphB receptors mediate cell migration and proliferation through P13K pathway and the protein kinase B/phosphatidylinositol 3-kinase (Akt/ PI3K) pathway is involved in the regulation of cell proliferation and viability. Eph/ephrin also regulates other signaling pathways including Abl/Arg and p53-family of tumor suppressor proteins [19]. A recent study in glioblastoma multiforme (GBM) shows that loss of EphA3 results in elevated MAPK signaling thereby inducing differentiation and reducing proliferation and self-renewal. This study showed that regulation of extracellular signal-regulated kinases/mitogen-activated protein kinases (ERK/MAPK) signaling by EphA3 is kinase independent of the upstream activators of MAPK signaling [22]. A similar finding has been made for GBM which express EphA2 at high levels [23].

In the ephrin-expressing cells reverse signaling can be induced after Eph/ephrin interaction [19,24]. Ephrin-B reverse signaling partly depends on the tyrosine phosphorylation of conserved residues in the cytoplasmic region, where phosphorylation is mediated by associated tyrosine kinases, most notably members of the Src family (Src, Fyn, Lyn, Yes) of tyrosine kinases. When the ephrin-B ligand is phosphorylated it can bind to cytosolic adaptor molecules via (Src Homology-2) SH-2 and SH-3 domains or PDZ domains [8]. The protein tyrosine phosphatase basophil-like (PTP-BL) has been identified as a negative regulator of ephrin-B signaling and it binds to ephrin-B through its C-terminal PDZ binding motif. De-phosphorylation of the ephrin-B cytoplasmic domain can inactivate the Src family of kinase and therefore cause termination of reverse signaling [25]. In the activated ephrin-B ligand, the PDZ motif plays an important role in assembly of other signaling

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molecules. Ephrin-B binds to cytoplasmic protein PDZ-RGS3, which contains a PDZ domain and a regulator of G-protein signaling (RGS) domain. Activation of the ephrin-B ligand by EphB receptor via the PDZ-RGS interferes with signaling of the stromal derived factor (SDF)-1 via its G-protein coupled receptor and the chemokine receptor-4 (CXCR4). Ephrin-B reverse signaling has thus been implicated with regulation of migration in cerebellar development [26].

The ephrin-A reverse signaling mechanism is not well understood, however it is likely that the signaling response is initiated by activity of the Src family of RTK in which transmembrane adaptor molecules, associating with the lipid anchor of the ephrin-A proteins, transmit the signal across the membrane. For example ephrin-A5 induces signaling within the ephrin-A expressing cell when bound to its cognate Eph receptor [27-29].

## Expression and Function of the Eph and Ephrin Genes

Eph signaling controls cell adhesion, migration, invasion and morphology by influencing integrin and intercellular adhesion molecule activity and by modification of actin cytoskeleton organization as described above. Through these mechanisms Eph function effects not only the processes of embryogenesis but also specialized cellular function in adult tissues, including bone remodeling, immune function and synaptic plasticity as well as cell proliferation and survival specific tissue stem cells as described further below [27,30].

Both Eph and ephrin proteins are important in regulating cell-cell interactions and their interaction can initiate either cell adhesion or repulsion. Cell repulsion occurs when bidirectional signaling triggers cytoskeletal contraction, loss of focal adhesions, cell rounding and cell detachment, whereas cell attachment occurs when signals are in favor of cell adhesion and migration [31]. This interaction influence many different cell behaviors during embryogenesis and in adult life [27]. Eph/ephrin interactions mediate formation of tissue boundaries (e.g. hindbrain rhombomeres) [32], control axon guidance during development and also tissue morphogenesis and patterning [33,34]. Eph/ephrin interactions are also involved in development of vascular system [35], stem cell biology [36], hematopoiesis, erythropoiesis, immune function and in tumor invasion and metastasis [27,30,37-39].

Many members of the Eph/ephrin family are expressed at high levels in some cancer cells and also elements of the tumor microenvironment, where they influence tumor growth and spread. Specific examples include lung, breast and prostate cancer, as well as melanoma, sarcomas and leukemias [40,41]. There is evidence that the Eph receptors can have either tumor suppressing or tumor promoting activity, depending on the tissue and their expression pattern [27]. Thus, the role and function of Eph/ephrins in cancer is not yet fully elucidated as some tumors show an elevated level of Eph expression while others show a decrease in Eph expression and as yet no single model of their function encompasses all cancers. For example EphA2 is up regulated in many cancers including breast and prostate and its expression is linked to an increase in malignancy [42,43] but it is down regulated in colon cancer [44]. Similarly, EphA1 expression is up-regulated in ovarian cancer [40] but down-regulated in advanced skin and colorectal cancers [45,46]. Studies have also shown the role of EphA7 as a tumor suppressor in follicular lymphoma [47], tumor suppressor function has also been reported for EphB receptors, including EphB2 and EphB3 in colorectal cancer [30,48]. EphB4 is another important example as this gene can act as either a tumor suppressor or an oncogene in different facets of breast cancer progression [49,50]. Table 1 represents the expression of Eph receptors in normal and malignant tissues [51-82].

## The Eph/ephrin in HSC and Leukemia

### Eph/ephrin expression in HSC and progenitors

The expression of Eph/ephrin has been detected on purified population of hematopoietic stem cells (HSCs) in both human and mouse. Gene expression analysis of HSC showed expression of ephrin-B2, indicating that it may be involved in signaling between HSC and their microenvironment. Other array based studies on primary human HSC ($CD34^+$ hematopoietic cells) shows expression of the EphA1 protein and its ligands ephrin-A3 and ephrin-A4 suggesting that their interaction may play a role in hematopoietic stem and progenitor cell positioning and function [83,84]. Further analysis of $CD133^+$ and $CD34^+$ hematopoietic stem cells in peripheral blood showed expression of EphA2 in all $CD34^+$ cells and the majority of $CD133^+$ cells however EphB2 was expressed in all $CD133^+$ cells and fifty percent of $CD34^+$ cells, these data suggest that a number of elements of the Eph/ephrin system may have a role in HSC function through regulatory effects on cell adhesion, migration and differentiation but also that there may be a degree of functional redundancy between several Eph proteins [85].

Real-time quantitative PCR of mouse $Lin^-ckit^+sca1^+$ (KLS) showed detectable expression of all EphA receptors except EphA6 and EphA8, along with ephrin-A ligands, with ephrin-A4 and ephrin-A5 being the most highly expressed ligands on purified HSCs in the mouse bone marrow [86]. Flow cytometric analysis of EphA2, A3, A4 and A5 along with ephrinA1-5 showed that EphA2 and EphA3 were the highest expressing EphA receptors. Expression of EphA2, A3, A4 and A5 was also detected in the mouse stromal cell lines however human stromal cell line showed only EphA2 expression at moderate levels [86]. Whilst the function has not been fully investigated, a role in HSC trafficking was demonstrated by treatment of mice with an Eph/ephrin inhibitor, EphA3-Fc, which resulted in mobilization of bone marrow progenitor cells into peripheral blood [86].

Some of the members of the Eph/ephrin family are also involved in development and regulation of mature hematopoietic cells. For example, EphA4 and EphB1 receptors along with ephrin-B1 ligand are expressed on human platelets [87], these studies also shows that EphA4 is involved in regulation of platelet aggregation and adhesion to fibrinogen, a process dependent on integrin $\alpha IIb\beta 3$ engagement [39].

EphB4 was originally identified on human bone marrow $CD34^+$ cells and its expression has been reported on erythroid progenitor cells in early stages of red blood cell development. Significantly, the EphB4 ligand, ephrin-B2, is expressed on bone marrow stromal cells [88] where it has been reported to be involved with regulating erythropoiesis via interaction with EphB4 [89]. Studies by Suenobu et al. [89] showed co-culturing hematopoietic progenitor cells expressing EphB4 with stromal cells expressing ephrin-B2 results in hematopoietic progenitor cells detachment from stromal layer and differentiation into a mature erythroid cells accompanied by EphB4 down regulation, however co-culturing these cell with ephrin-B2-negative stromal cells resulted in less maturation of erythroid cells and no change to EphB4 expression [89]. Ephrin-B2 ectopic expression in stromal cells increased adhesion of hematopoietic cells to stromal calls and decreased transmigration of hematopoietic cells beneath a stromal cell monolayer. These findings strongly support a role for the EphB4/ephrin-B2 interaction in migration and colonization of stem/progenitors cells in the bone marrow microenvironment [90].

Some of the Eph/ephrin molecules are also involved in lymphoid development. The expression of Eph/ephrin has been studied

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| Eph | Tissue expression | Expression in cancer | References |
|---|---|---|---|
| EphA1 | Widely expressed in mouse epithelial tissues evidence of expression in hematopoietic progenitors | Over expressed in many different cancers including hepatocellular, prostate, lung, gastric and colon cancer<br>Down regulated in non-melanoma skin cancer, colorectal cancer | 1,12,24,45,46,51-55 |
| EphA2 | Expressed highly in adult human epithelial cells and endothelium | Over expressed in prostate, breast, melanoma, lung and ovarian cancers<br>Up-regulated in glioblastoma<br>Down-regulated in colon cancer | 23,24,42,43,54-61 |
| EphA3 | Expressed in various stages of embryonic development and in adult central nervous system | Expressed in neural cancers, leukemia, lymphomas and sarcomas<br>Up-regulated in lung, brain, liver and kidney<br>Over expressed in melanoma | 24,54,55,62-66 |
| EphA4 | Expressed in development, final stages of embryogenesis and central nervous system | Expressed in prostate, pancreatic cancer<br>Up-regulated in lung cancer<br>Down-regulated kidney<br>Over expressed in gastric cancer | 24,54,55,62,67-70 |
| EphA5 | Expressed in nervous system | Expressed in neuroblastomas and neural cancer<br>Down-regulated in breast cancer | 24,54,55,70-72 |
| EphA6 | Expressed more prominently in adult tissues than in embryonic tissues | Down-regulated in colon cancer and renal carcinoma<br>Up-regulated in lung and liver cancer | 24,54,55 |
| EphA7 | Expressed in developing neural tubes, thymus, lymphoid tissues and fetal bone marrow | Expressed in colorectal cancer, lung and follicular lymphoma<br>Up-regulated in ALL1 leukemia | 24,44,47,54,55,73-75 |
| EphA8 | Expressed in spinal cord and neuronal cells | Expressed in colon cancer<br>Down-regulated in glioblastoma | 24,54,55 |
| EphA10 | Expressed in testis | Over expressed in breast cancer | 76,77 |
| EphB1 | Expressed in Brain and colon | Expressed in lung cancer<br>Down-regulated in colon carcinoma and in kidney cancer | 24,46,54 |
| EphB2 | Expressed in epithelial cells, thymus, lymphoid, osteoblastic and osteoclastic cells | Over expressed in gastrointestinal<br>Expressed in colon, ovarian and lung cancer<br>Up-regulated in colorectal, kidney and hepatocellular cancer | 24,54,78 |
| EphB3 | Expressed in various tissues | Expressed in prostate, lung and melanoma | 24,54 |
| EphB4 | Expressed in placenta and in range of primary tissues including brain, endothelium, hematopoietic cells | Expressed in colon, endometrial, breast, neuroblastoma, glioblastoma and leukemia and lymphoma cancer | 24,54,79-81 |
| EphB6 | Expressed in various tissues including brain, pancreas, thymus and T-cells | Expressed in T cell tumors and leukemia<br>Up-regulated in colon cancer<br>Down-regulated in breast, lung and kidney cancer | 24,54,82 |

**Table 1:** Eph expression in normal and malignant tissues.

extensively in T-lymphocytes and expression of some of the members of this family including EphA1, EphA2, EphA3, EphA4, EphA7, EphB2, EphB6, ephrin-A1 ephrin-A3, ephrin-A5 and ephrin-B1 has been reported in the thymus, both on thymic stroma and lymphoid cells suggesting a role in T-cell development [91-93]. Interestingly, there are no reports of defective T lineage development in knockout mice, perhaps a result of there being multiple Eph receptors with overlapping functions in the T cell compartment.

As well as T-lymphocytes the expression of Eph/ephrin gene has been also reported in B-lymphocytes [38,94,95]. EphA7 and EphA4 transcripts were found in human fetal bone marrow pro-B and pre-B cells. EphA4 expression is found in both adult and fetal pro-B and pre-B lineage cells with high levels of expression in peripheral blood. The full length EphA7 transcript, however, was not found in mature fetal B-lineage and adult B-lineage cells. This suggests that EphA7 may be involved in expansion and /or differentiation of pre/pro B-cell but is lost on mature B cells [95]. Further studies show that there are different types of EphA7 mRNA, one of which encodes the full length EphA7 and another splice variant encodes a truncated, soluble protein that lacks the cytoplasmic domain. Studies by Dawson et al. [74] showed that normal lymphocytes express and secrete the truncated form of EphA7 [74], which have been shown to have tumor suppressive effects in lymphoma [47].

## Eph/Ephrin in vascular development

In the context of hematopoiesis, the vascular system is crucial in development of blood cells within the bone marrow and also in the function and migration of mature hematopoietic cells. The vascular system arises from two distinct processes known as vasculogenesis and angiogenesis. Vasculogenesis is an early event in embryonic development involving mesodermal cells differentiation to form a vascular plexus in embryonic tissues and angiogenesis is a process in which new blood vessels are formed from existing blood vessels. Angiogenesis has a role both in early development and in the adult hematopoietic system, it also promotes sprouting of new blood vessels in embryonic and postnatal vasculature and it has been shown to be important in development and metastasis of solid tumors [96]. Some members of Eph/ephrin family have established roles in vasculogenesis and angiogenesis. The expression analysis of Eph/ephrin using real-time polymerase chain reaction (RT-PCR) has shown expression of EphB2, EphB3, EphB4, ephrin-B1 and ephrin-B2 in the yolk sac [97]. Ephrin-B1 expression has been detected on both arteries and veins while the high affinity ligand for EphB4, ephrin-B2, is only detected on arteries and EphB4 expression is only detected on veins. Knock out ephrin-B2 mice and some of the EphB2 and EphB3 double mutants mice have defects in embryonic vasculature and therefore these mice are embryonically lethal. EphB2 and ephrin-B2 expression in mesenchyme adjacent to vessels and the vascular defects in EphB2/EphB3 double mutants indicate a requirement for Eph/ephrin signaling between endothelial cells and surrounding mesenchymal cells [97]. EphA2 has also been reported to have a role in angiogenesis and the expression of EphA2 and its ligand ephrin-A1 has been reported in both human and mouse tumor vasculature [61].

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### Eph/Ephrin on bone remodeling and formation

The bone marrow is the principal site of hematopoiesis in adult animals and requires both vascular and other stromal cell types to create the hematopoietic niche. Important amongst these are the osteoblasts and osteoclasts, which mediate bone formation and remodeling. Bone is constantly remodeled through resorption of mineralized bone by osteoclast and formation of new bone by osteoblast. Coupling of bone resorption and formation is critical during normal bone remodeling and it is necessary for bone growth, any deregulation in this process will result in pathological bone disease [19,78].

Eph receptors and ephrin ligands are important in bone remodeling and homeostasis during this process Eph/ephrin bidirectional signaling regulates differentiation and function of the bone cells. Real time PCR (RT-PCR) analysis of the Eph/ephrin showed mRNA expression of ephrin ligands, ephrin-B1, ephrin-B2 and ephrin-A1, A2, A4 and A5, as well as Eph receptors including EphB2-4, EphB6, EphA2-4 and EphA7 receptors on osteoblastic and osteoclastic cells [98-100]. Expression of EphB4 is observed on the osteoblasts and forward signaling through EphB4 results in bone formation and reverse signaling through ephrin-B2 inhibit bone resorption therefore ephrin-B2/EphB4 act as coupling stimulator [98]. Expression of ephrin-A2 was observed during early osteoclastogenesis and unlike the ephrin-B2 it acts as coupling inhibitor as reverse signaling through ephrin-A2 result osteoclastogenesis and EphA2 forward signaling into osteoblast inhibit osteoblastic bone formation and mineralization [99]. Ephrin-A2/EphA2 bidirectional signaling facilitates bone remodeling at initiation phase, forward signaling through EphA2 receptor on osteoblast inhibit osteoblastic differentiation and bone formation and reverse signaling into osteoclast through ephrin-A2 promote osteoclast differentiation [78,99]. Ephrin-B1 full knockout mice are prenatally lethal and they have skeletal defects. Studies on disruption of ephrin-B1 on collagen I producing cells result in reduced bone formation and skull defect and studies on ephrin-B1 conditional knockout mice shows defects in osteoblastic mediated bone formation with no increase in osteoclastic bone resorption and this condition results in reduction in bone size and density [101,102].

The importance of Eph/ephrin interactions has also been shown in various stem cell niches, including neural, dental and intestinal stem cell compartments [103,104]. More recent studies show their involvement in bone homeostasis and mesenchymal stem cell (MSC) regulation. Arthur et al. [104] showed increase in osteogenic differentiation upon ephrin-B1 and/or ephrin-B2 expression by MSC. They also showed that ephrin-B1 activation promoted chondrogenic differentiation; therefore EphB/ephrin-B interactions may be involved in recruitment, migration and differentiation of MSC during bone repair [104]. Studies by Ting et al. [86] shows that ephrin-A signaling interact with stem/progenitor cells in the bone marrow niche as it's signaling mediates the release of progenitor cells from hematopoietic niche [86].

Interestingly, Eph/ephrin interactions are also involved in bone malignancies and tumors, osteocarcinoma is a malignant bone tumor in adolescence and microarray analysis studies show increased expression of EphA2, EphA4, ephrin-B1 and ephrin-A1 in osteosarcoma cells [105,106].

### Eph/Ephrin expression in leukemia and other hematopoietic tumors

Both chronic and acute myeloid leukemia are malignant diseases of the hematopoietic system which in most cases are believed to arise through the abnormal proliferation of either uncommitted or partially committed HSC [107]. The origin of other types of leukemia such as promyelocytic leukemia, pre-B acute lymphoblastic leukemia (ALL) and T-ALL and chronic lymphoblastic leukemia (CLL) are more likely due to malignant transformation of more mature progenitor cells. Expression of elements of the Eph/ephrin system has been detected on many types of human leukemia. One of the best studied is EphA3, which was originally identified in the LK63 pre-B ALL cell line and further investigations revealed its expression in T-cell leukemia cell lines such as Jurkat, JM and HSB-2 [65,108]. It has been shown that EphA3 can induce both adhesive and cell repulsive responses in different cell types [109]. In analyzing ephrin induced cell adhesion in LK63 cells, a critical role was identified for protein phosphatase activity, which prevented EphA3 phosphorylation and hence maintained the Eph/ephrin adhesive bond and prevented initiation of the signaling mechanisms leading to cell repulsion [31]. In leukemia, EphA3 is expressed at significantly higher levels compared to normal blood cells, elevated expression of EphA3 being detected in a proportion of clinical samples from cases of lymphoid and myeloid leukemias [111]. Elevated EphA3 expression has also been detected on other cancers such as lung cancer, melanoma and brain tumors [22,64,65,109], whereas expression was found to be absent or low in corresponding normal tissues [54,65,110]. Recent array based studies also showed EphA3 as one of the genes with copy number alteration (CNA) in the genome of acute myeloid leukemia (AML) patients [111]. Further studies by Guan et al. [112] showed copy number variation (CNV) of EphA3 to be associated with various types hematological malignancies and therefore CNV of EphA3 could be used as a diagnostic indicator for different types of leukemia [112]. Many cancers, including leukemia, require multiple cooperative oncogene mutations for malignant cell transformation. Specific sets of synergistically dysregulated by cooperative oncogenes are known as cooperative response gene (CRGs), which regulate leukemia stem cell (LSC) growth and survival. Studies by Ashton et al. [113], where stem cells were retrovirally transduced with two fusion genes found in human myeloid leukemias. NUP98-HOXA9 and BCR-ABL, have identified EphA3 as a common CRG. They showed that shRNA knock down of EphA3 in leukemic stem cells reduced leukemic cell engraftment, concluding that this gene may be responsible for leukemia stem cell growth and survival in bone marrow microenvironment [113]. With the involvement of EphA3 in many different types of leukemia a high affinity monoclonal antibody to EphA3 (IIIA4) [65] has been fully humaneered by Kalabios and the resulting antibody, KB004, is now in phase I clinical trial in leukemia and other hematological cancers [114].

As mentioned previously EphB4 was originally identified in human bone marrow $CD34^+$ cells and its expression been reported in erythroid progenitor cells, however it's ligand ephrin-B2 is expressed in bone marrow stromal cells [88]. Co-expression of EphB4 and ephrin-B2 is found in the yolk sac, which is the first site of hematopoiesis and vascular development during embryogenesis. EphB4/ephrin-B2 expression has been shown in the majority of leukemia and lymphoma cell lines although expression in clinical samples appears less prominent [81]. Antibodies to EphB4 have undergone extensive pre-clinical evaluation and shown good anti-tumor effects in solid tumors, which over-express EphB4 and by inhibition of angiogenesis, although no efficacy has been shown in hematopoietic tumors to date [115]. Nevertheless, these antibodies may have the potential to be developed to target EphB4 in leukemia and related blood cancers.

Studies by Nakanishi et al. [75] shows EphA7 up regulation in the ALL1 associated leukemia (ALL1/AF4 and ALL1/AF9). They also showed that EphA7 up-regulation was associated with phosphorylation

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| Eph | Targeted therapy | References |
|---|---|---|
| EphA1 | Tumor suppressor in colorectal cancer | 44 |
| EphA2 | EphA2 targeting reagents in ovarian cancer therapy | 119 |
| EphA3 | Therapeutic target in leukemia and glioblastoma | 22,110 |
| EphA7 | Tumor suppressor in T-LBL and follicular lymphoma | 47,120 |
| EphB4 | EphB4 antibody to inhibit solid tumor growth | 115 |

**Table 2:** Eph receptors as a therapy target for cancer.

of ERK and treatment with a phosphorylated ERK blocking drug resulted in apoptotic cell death in ALL1/AF4 leukemic blast cells [75]. Thus, anti-EphA7 antibodies or other inhibitors may well have a role in leukemia associated with this translocation. In contra-distinction to this positive role in leukemia, EphA7 is lost in lymphomas, where the gene is hypermethylated and repressed in germinal center B-cell non-Hodgkins lymphomas and this has a potential to influence tumor progression and spread [74]. In this study a soluble form of EphA7 was shown to inhibit lymphoma in a mouse model, a chimeric protein consisting of soluble EphA7 and CD20 antibody had still greater therapeutic effect. Further studies show EphA7 as targeted tumor suppressor gene in T-cell lymphoblastic leukemia and lymphoma (T-LBL) and follicular B cell lymphoma [47,116].

EphB6 expression has been observed in normal human tissue and over-expression of EphB6 has also been reported in both myeloid [117] and lymphoid leukemias [82,118]. The expression level of EphB6 decreases with maturation of the cells in T-cell derived leukemia-cells, therefore suggesting that EphB6 expression regulates T-cell development but has less significant role in mature T cells [82]. To date there are no reports of experimental therapies targeting EphB6. Table 2 [110,119,120] represents the summary of Eph receptors used as therapy target in various malignancies.

## Summary

In summary, the aberrant expression of Eph receptors in hematopoietic tumors reflects the spectrum of functions of these receptors in all cancers. In some cancers these genes act as tumor suppressor, examples being EphA1 in colorectal cancer and EphA7 in follicular lymphomas. On the other hand these proteins can also have oncogenic effects, examples being the expression of EphA2 and EphA3 in glioma and the over-expression of EphA3 in leukemia. In terms of therapy, the over-expression in certain tumors, taken together with the surface expression of these proteins, makes a strong case for targeted therapies. This is particularly the case when expression on normal tissues is minimal; this is exemplified by EphA3 and EphB4 where no toxicity was evident in pre-clinical testing of potential therapeutic antibodies. These studies reveal the therapeutic potential of targeting components of the Eph/ephrin system in leukemia and other cancers. These results should prompt further research into the specific roles of these proteins in different cancers as a prelude to designing and optimizing the therapeutic targeting of these proteins.

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